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Annual Report of
Intramural Research Program Activities

National Institute on Alcohol Abuse and Alcoholism

Fiscal Year 1991

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
Public Health Service
Alcohol, Drug Abuse, and Mental Health Administration

Administrative Document

Annual Report of Intramural Research Program Activities

National Institute on Alcohol Abuse and Alcoholism

October 1, 1990 to September 30, 1991

Summary Statements and Individual Project Reports

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
Public Health Service
Alcohol, Drug Abuse, and Mental Health Administration
National Institute on Alcohol Abuse and Alcoholism
9000 Rockville Pike
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Annual Report of the
Division of Intramural Clinical and Biological Research
National Institute on Alcohol Abuse and Alcoholism
October 1, 1990 - September 30, 1991
Markku Linnoila, M.D., Ph.D., Director

FY 1991 has seen the NIAAA Intramural Research Program (IRP) in a relative holding pattern personnel and resource wise, but scientifically the Program, as a whole, has been extraordinarily productive. Both the basic and clinical resources allocated to the program have been effectively utilized, and significant new insights have been gained to the many mechanisms of ethanol's actions. Parts of the Program have gained international recognition in the form of invited lectures overseas at major international meetings. This has been especially true for the work performed on ion channels, mass spectrometric analyses of cell membrane phospholipids, alcohol abuse in rhesus monkeys, and alcohol and violence in humans.

Investigators in the IRP eagerly anticipate the opening of Building 49 in FY 1992 because the laboratories of the IRP are severely overcrowded.

Collaborations with other Institute's Intramural Research Programs on the NIH campus have been extensive, particularly in the area of molecular genetics and clinical research. Intra- and extramural cooperation has been very extensive in the realm of the medications development intiative, and in composing NIAAA initiatives for the NIH strategic planning process.

The major research themes of the IRP in FY 1991 have included brain imaging using both position emission tomography (PET) to quantify neuronal activation within anatomically well-defined brain areas as determined by magnetic resonance imaging. Applying these dual technologies to investigate changes in the metabolic state of the brain during craving for alcohol is expected to yield information which is of fundamental importance to understand the process of relapse in patients with alcohol dependence.

Studies on effects of ethanol on cell membrane receptors, ion channels, and expression of genes coding for these important proteins are yielding fundamental insights into basic mechanisms of ethanol's action. Combined with studies on region specific effects of ethanol on the release of neurotransmitters, these investigations will elucidate how ethanol produces reward, dependence, tolerance and brain damage. Behavioral studies on whole animals, using primarily mice and monkeys, combined with molecular genetics and behavioral manipulations during development, examine important protective and causal factors for alcohol abuse and dependence. These investigations have already pinpointed developmental factors in rhesus monkeys which are conducive of alcohol abuse in this species.

Studies into the effects of ethanol on the regulation of immune function and blood pressure regulation have revealed ethanol-induced changes in important regulatory functions conducive of increased risk of various infections, including AIDS, and cerebrovascular incidents, such as strokes, in alcoholics.

All in all, the Intramural Research Program is a creative, vibrant, internationally known and highly productive research center which conducts studies at the cutting edge of science in several fast developing fields.

Annual Report of the
Laboratory of Clinical Studies
Division of Intramural Clinical and Biological Research
National Institute on Alcohol Abuse and Alcoholism
October 1, 1990 to September 30, 1991
Michael J. Eckardt, Ph.D., Chief

Introduction

During Fiscal Year 1991, investigators in the Laboratory of Clinical Studies (LCS) continued conducting the projects outlined in previous annual reports and initiated new projects to insure diagnostic accuracy (including Axis II), investigate the utility of novel measures of liver function, and expand the studies of physiological and psychological mechanisms involved in impulse control disorders associated with excessive alcohol consumption. Continued emphasis remained on the genetics of alcoholism and related disorders and on biochemical and genetic concomitants of impulsivity and violent behavior. Special projects concerning American Indians, Blacks, women, and violent Finns have continued with increased intensity. The Laboratory continues investigating the effects of excessive alcohol consumption on the central nervous system and on physiological complications of alcohol abuse and dependence. It is also testing novel approaches to improve the maintenance of abstinence in alcohol dependent patients.

The past year has been administratively difficult, with the loss of two senior scientists and a greater than expected turnover of clinical associates. Moreover, the uncertainty associated with the appointment of the new Scientific Director and resultant selection of an Acting Chief of LCS has decreased research capability and long-range planning. In spite of these difficulties, LCS has maintained research productivity and has initiated a number of new studies.

I. Section of Clinical Science

The main objectives of the research conducted in this Section are to 1) characterize the role of various neurotransmitter systems in the etiology of alcoholism by utilizing cerebrospinal fluid metabolite determinations, pharmacological challenge paradigms, and PET scans; 2) explore possible biochemical determinants that might differentiate subtypes of alcoholic patients; 3) describe and understand the behavioral and biochemical interactions between alcoholism, panic disorder, and depression; 4) explore sex differences in alcoholism by using a serotonin challenge paradigm; 5) probe the role of diet, and carbohydrate consumption in particular, as a possible determinant of alcohol ingestion; 6) employ procaine as a probe for limbic system function in alcoholics with and without panic disorder; and 7) introduce new pharmacological interventions for long-term treatment of alcoholism.

Findings:

Alcoholics with an early onset of drinking-related problems (Type II) are statistically more likely to "desire a drink of alcohol" following IV administration of the serotonergic partial agonist, m-chlorophenylpiperazine (m-CPP), than late onset alcoholics (Type I) or controls. Preliminary results obtained from m-CPP administration in women indicate that their behavioral responses are similar to those of Type I male alcoholics. Studies exploring the functional role of m-CPP in the central nervous system (CNS) are currently underway using deoxyglucose PET scans during m-CPP challenge. Demographic studies indicate that patients with an early onset of alcoholism have an increased frequency of other psychiatric disorders, as well as lower levels of CSF diazepam binding inhibitor and somatostatin concentrations when compared to alcoholics with an onset of problems after 20 years of age.

The administration of sodium lactate revealed that detoxified alcoholics with panic disorder are less likely to experience a panic attack than similarly challenged nonalcoholic patients with panic disorder. This suggests that panic attacks in alcoholics may have a different biochemical etiology than in non-alcoholics with panic disorder. Currently, the role of glucose in blocking lactate-induced panic attacks is being assessed. A study examining the CSF from alcoholic patients with panic disorder showed that they had higher levels of endorphins than alcoholics without panic disorder.

2-deoxyglucose (2-DG), a glucose analogue which competitively inhibits glucose-6-phosphate dehydrogenase and leads to intra-cellular glucoprivation, was administered to alcoholics and controls. Preliminary analyses indicate that alcoholics consumed less calories than controls following both 2-DG and placebo. However, alcoholics consumed more carbohydrates than controls. No desire to drink alcohol was reported as a result of the 2-DG administration.

Preliminary results show that there are statistically significant differences among controls, alcoholics and alcoholics with panic disorder in the behaviorally-induced effects of procaine. Alcoholics with panic disorder were more likely to experience a panic attack following procaine than were alcoholics without panic disorder or controls. Alcoholics with panic disorder had a greater rise in pulse per unit time and a greater change in vagal tone when compared to either controls or alcoholics without panic disorder.

To date, 28 alcoholic patients are enrolled in a protocol assessing the effectiveness of buspirone (a selective serotonin agonist) vs. placebo on reducing alcohol consumption in recovering alcoholics. Data analyses will begin when sufficient patients have been enrolled in the study.

Future studies:

Studies are planned to investigate 1) the feasibility of combining smoking cessation with alcohol cessation and 2) to study the phenomena of "being out of control" as it pertains to panic, aggression and alcoholic drinking.

II. Unit of Family Studies

The Family Studies Program clinically evaluates subjects for genetic, physiological and biochemical studies conducted by other investigators in the Laboratory as well as studies within the Unit itself. Research currently in progress includes correlational studies comparing different subgroups of alcoholics, in order to elucidate risk factors for alcoholism and impulsive behaviors such as suicide attempts, physical violence and drug abuse. There is an additional focus on clinical personality traits within these subgroups. A study comparing offspring of middle-class, Black and Caucasian alcoholics and those of nonalcoholics is in progress. The Unit is also in the process of behaviorally phenotyping large pedigrees of alcoholic American Indians, Blacks and Caucasians in collaboration with the Section of Genetic Studies.

III. Section of Genetic Studies

To identify genetic loci determining alcoholism, the Section of Genetic Studies is testing for linkage or association between genetic markers and alcoholism and related behavioral phenotypes, and it is engaged in molecular cloning studies on genes related to serotonin function and alcohol metabolism. These studies involve the use of cloning, sequencing and genetic expression methods to analyze behaviorally significant genes such as tryptophan hydroxylase, which is rate limiting for the synthesis of the neurotransmitter serotonin, and genes coding for serotonin receptor and uptake proteins. By isolating the tryptophan hydroxylase cDNA and gene, its location on mouse chromosome 7 was established, putative regulatory sequences were delineated and an abundant polymorphism was found. Our cloning of this enzyme gene is allowing us to pursue a variety of questions regarding the regulation of serotonin synthesis and genetic variation in serotonergic function. Isolation of the human class III alcohol dehydrogenase cDNA has enabled the analysis of its structure and tissue distribution, and we are now pursuing the possible relationship of this enzyme, which has an important role in one carbon metabolism, to genetic disease.

In the area of human behavioral genetics, suitable families and populations are being studied using techniques for genetic linkage and transmission analysis. For example, the widely publicized strong linkage of the dopamine D2 receptor gene to alcoholism in the general population was shown to be nonreplicable in a population of carefully characterized Caucasian alcoholics, in two extended families and in impulsive Finnish alcoholics. To better understand the genetic underpinnings of behavior, this Section is focusing efforts on relatively homogeneous populations: impulsive and violent Finns, American Indians, and families intensively trait-phenotyped in collaboration with other LCS staff. A large number of dispersed polymorphic markers are being applied, and we have developed polymorphisms at several candidate loci, including TrpOHase, the 5HT1A serotonin receptor and the GABA B1 benzodiazepine receptor. These markers are also being applied in linkage studies on families with the low voltage alpha genetic variant of the EEG. We have shown that the low voltage alpha variant is increased in frequency in alcoholism and anxiety and confirmed that it is transmitted in autosomal dominant fashion.

IV. Section of Clinical Brain Research

Investigators in the Section of Clinical Brain Research conduct sophisticated electrophysiological, neuropsychological, and brain imaging studies on alcoholics, individuals at risk, and carefully-matched controls. Brain electrical and autonomic nervous system measures of alerting and orienting are enhanced by alcohol, in contrast to its depressant effects on most other functions. A similar effect was observed in a sample of alcoholic organic brain disorder patients. Such findings suggest that alcohol intoxication and alcoholism-associated organic brain disease may be associated with a disinhibition or deregulation of orienting processes. Advances in brain imaging include (1) development of two-dimensional registration and rescaling algorithms, which enable group averaging and increase the signal-to-noise ratio, and (2) application of frequency analysis to study the basic underlying informational patterns embedded in the brain images.

Acute effects of alcohol and other drugs on memory functioning continues to be studied and compared to the more severe deficits observed in chronic alcoholics. A study comparing memory functioning in patients with alcohol amnestic disorder, unipolar depression, Alzheimer's dementia, and controls has been completed. A large (n=100) study has recently been completed in young alcoholics (18-35 years of age) where cognitive function is related to family history of alcoholism, hyperactivity/learning disabilities when young, and recent and chronic alcohol consumption. In addition, animal studies are still being conducted on the behavioral effects of alcohol and alcohol antagonists.

V. Section of Clinical Biochemistry and Pharmacology

Attempts to develop a noninvasive test of liver function based on measurement of labeled carbon dioxide in breath after administration of 13C labeled drugs were hindered by the lack of substrates with suitable sensitivity and the project has been terminated. The interests of the Section have now changed with the hiring of new scientific personnel. Research efforts in the Section of Clinical Biochemistry and Pharmacology will be concerned with exploring the mechanisms responsible for the altered rates of drug metabolism seen in abstinent alcoholics. Literature searches suggest that the activities and levels of cytochrome P450 may be altered in alcoholic patients. Initial studies in rats will guide the development of human research protocols. Initial research will focus on the evaluation of cytochrome P450 isozymes and membrane structure in rats during ethanol exposure and following ethanol withdrawal.

VI. Section of Analytical Chemistry

Research efforts in the Section of Analytical Chemistry are focused upon the alterations in biological functions in cell membranes associated with alcoholism with particular reference to the modification of polyunsaturated lipid metabolism and composition. Emphasis has been placed on essential fatty acid composition and metabolism as well as membrane localization and associations of polyunsaturated phospholipids. Our previous studies have indicated that alcohol leads to essential fatty acid decline in many tissues in an animal model of chronic alcohol abuse. In extending these animal studies, it was found that a borage oil based diet, rich in 18:3n6, was able to partially protect the liver from alcohol-induced loss of arachidonic acid (20:4n6). A clinical study of alcoholics has been carried out in this reporting period in order to generalize these results to humans. There were losses of n-6 essential fatty acids in both platelets and erythrocytes in these patients and, in most cases, there was a replacement of these lipids with non-essential species. In the case of the erythrocyte arachidonic acid, the metabolic picture was more complex as this precursor for eicosanoids actually increased in one phospholipid class while decreasing in another. After four weeks of abstinence, there was little or no recovery in the lipid species that were abnormal at admittance. It was also shown that the aminophospholipid molecular species asymmetry increased for most species in the erythrocyte plasma membrane. At present, an effort is being made to correlate these findings with the dietary histories of these patients and the essential fatty acid intake during hospitalization. These were the first studies of phospholipid molecular species composition and asymmetry in the field of alcoholism. A new initiative has been started that will explore the consequences of polyunsaturate deficiencies in terms of membrane functions of polyunsaturated phospholipids, their biophysical properties and their associations with proteins.

Progress has been made in characterizing the enzymatic oxygenation of both n-3 and n-6 families of highly unsaturated fatty acids and in defining the effects of ethanol on these processes. We have now clearly shown using stereochemical analysis that rat brain does not have a lipoxygenase except in the pineal gland. Pineal 12- or 15-lipoxygenases are able to use either 20:4n6 or 22:6n3 as substrates although the latter is the preferred substrate for the 15-LO. There are apparently 15-lipoxygenase products endogenous to this gland and their levels are decreased by ethanol exposure. This occurred in spite of a stimulation of phospholipase A2 by ethanol which would be expected to release more substrate for this enzyme. The depression of lipoxygenase activity by ethanol was also demonstrated for ionophore-stimulated activity on endogenous substrate in the platelet. The biological activity of the lipoxygenase products of 22:6n3 has been further elucidated with the finding that they lead to an increased release of leukotrienes in lung, neutrophils and monocytes. Since they depress prostanoid release at the same time, the mechanism of lipoxygenase stimulation may be through the "alternate pathway mechanism".

Rapid progress has been made in the development of an in vivo method for fatty acid elongation and desaturation assay in mammals. A gas chromatography-mass spectrometry technique using deuterium- labelled fatty acids, derivatization with electron-capturing fluorinated compounds and the use of the negative ion mode has made possible a three orders of magnitude increase in method sensitivity. Since the method is non-toxic and non-invasive, it has direct application to human subjects. The analytical part of this project has been completed and in vivo work in rats, cats and rhesus monkeys begun. Both dietary variables such as the type of fat in the diet and acute or chronic alcohol exposure are being studied with this paradigm. It is now possible to apply this approach to human subjects and to test the notion proposed in the 1970s that alcohol inhibits liver desaturases. It also provides great promise as an excellent diagnostic assay for liver function that will be of great utility for alcoholics and others with liver disease.

Publications
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October 1, 1990 to September 30, 1991

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AA 00257-07 LCS

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Neuroendocrine Studies in Offspring of Familial Alcoholics

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	G. Brown	Unit Chief	LCS, NIAAA
Others:	S. Goodson	Senior Staff Fellow	LCS, NIAAA
	M. Linnoila	Scientific Director	NIAAA

COOPERATING UNITS *(if any)*
None

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0.5	0.25	0.25

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☒ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Responses of thyroid stimulating hormone (TSH) to thyrotropin releasing hormone (TRH), have been studied in the offspring of familial alcoholics and age, sex, and past alcohol exposure matched control children. Sons but not daughters of familial alcoholics were found to have exacerbated TSH responses to TRH infusions.

Project Description:

Investigators:

G. Brown	Unit Chief	LCS, NIAAA
S. Goodson	Senior Staff Fellow	LCS, NIAAA
M. Linnoila	Scientific Director	LCS, NIAAA

Objectives:

We are investigating the persistently blunted TSH response to TRH infusions in long-term abstinent alcoholics, described by Larsen and Prange, as a possible genetic marker for vulnerability to alcoholism.

Methods Employed:

In the preliminary study, fifteen sons and fifteen daughters of familial alcoholics and 30 matched control children received i.v. infusions of TRH while at bed rest in our Outpatient Clinic, Triiodothyronine, thyroxine, and TSH concentrations were quantified. The sample has been expanded to 77 subjects at the present time. We are aiming at a sample of 120 subjects to provide a definitive finding. This sample size was deemed necessary by statistical power calculations.

Major Findings:

Sons, but not daughters, of familial alcoholics had markedly higher TSH responses to TRH than their matched controls. No differences were found in prolactin growth hormone and T3 responses between the children at risk for becoming alcoholics and controls.

Significance to Biomedical Research and the Program of the Institute:

This is potentially the first male-limited neuroendocrine abnormality described in children of familial alcoholics. If confirmed in further studies, an exacerbated TSH response to TRH infusions may become useful for identifying individuals at a high risk of becoming alcoholics. Furthermore, the finding may be indicative of a primary serotonergic deficit in boys at high risk of becoming alcoholics.

Proposed:

Two problems have prevented the completion of this project: lack of a physician to run the study and an appropriate group of children. It appears that both of these problems are resolved at present and we would expect to complete the study.

Publications:

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AA 00276-03 LCS

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Psychobiology and Behavior of Aggression and Suicide in Adults and Children

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	G. Brown	Unit Chief	LCS, NIAAA
Others:	S. Goodson	Senior Staff Fellow	LCS, NIAAA
	F. Goodwin	Administrator	ADAMHA
	M. Linnoila	Scientific Director	NIAAA

COOPERATING UNITS *(if any)*

Office of Administrator, ADAMHA (F. Goodwin)

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 ☒ (a1) Minors
 ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Studies that relate human (including Disruptive Behavior Disorders in children) aggression and suicide to various behavioral and biological factors have been ongoing. The most significant finding is a trivariate relationship among history of aggressive behavior, history of suicidal behavior, and low cerebrospinal fluid (CSF) 5-hydroxyindoleacetic acid (5HIAA). The data indicate that certain aggressive, impulsive, and depressive characteristics in childhood are inversely related to CSF 5HIAA measured during late adolescence; family instability (particularly alcoholism in a parent) during childhood is also associated with an increased likelihood of aggressive and suicidal behavior in adolescence. Offspring of parents with major affective disorders are more likely to manifest suicidal behavior as adolescents than offspring of control parents. These data, along with the work of other investigators studying aggressive and depressive behavior in childhood, indicate the possibility of traits associated with disordered serotonin metabolism; further, the less consistent relationship between lower CSF 5HIAA and suicidal behaviors vs. aggressive behaviors, may indicate that some suicidal behaviors are a self-destructive manifestation of a more basic destructive (aggressive/impulsive) trait.

Project Description:

Investigators:

G. Brown	Unit Chief	LCS, NIAAA
S. Goodson	Senior Staff Fellow	LCS, NIAAA
F. Goodwin	Administrator	ADAMHA
M. Linnoila	Scientific Director	NIAAA

Objectives:

The objective is to gain new knowledge of the central nervous system (CNS) of children, adolescents, and adults with reference to maturational changes and neuropsychiatric disorders as they relate to aggression and suicide. Alterations in neurotransmitter metabolism have been proposed for children and adolescents who have psychiatric diagnosis within the DSM-III-R Disruptive Behavior Disorder (DBD) group, VIZ. DMS-III-R diagnoses: Attention Deficit Disorder [ADD]; Conduct Disorder [CD]; and Oppositional Defiant Disorder [ODD]. Amine systems have also been linked to adult psychiatric illness. Searching for interrelationships between neurochemical functioning and repeated behavioral patterns may be as important as searching for biochemical trails related to traditional diagnosis. Direct human data can be valuable in assessing differences and similarities between humans and animals. Data now relate CNS function to some behaviors associated with personality disorders; i.e., aggressive-impulsive, obsessive-compulsive, alcoholic, etc. Aggressive/impulsive characteristics have been linked to a genetic predeterminant of suicidal behavior independent of psychiatric diagnosis. Animal data strongly suggest relationships between aggressive behavior and neurotransmitters, particularly (5HT). A purpose of this project is to extend studies of CNS amines into larger and more diverse groups.

Methods Employed:

In- and outpatient programs have involved children and adults. Offspring of normal parents and parents with a history of affective disorder have been studied; offspring of parents with a history of alcoholism are being studied. Children may be evaluated medically, psychiatrically, and psychometrically, including clinical procedures and laboratory studies. Details can be found in NIMH protocols #85-M-115 and #79-M-123. Previous NIMH-NNMC studies have been described (Z01 MH 00092-11 BP). Among individuals incarcerated for murder on whom baseline cerebrospinal fluid (CSF) is available, psychological responses to the glucose tolerance test will be assessed by the Thematic Apperception Test; measures of Y-chromosomal material are being studied (Linnoila et al.). These studies have been extended to children and families. Further, areas of behavioral disturbance thought to be associated with 5HT dysfunction in children are being assessed, e.g. aggressive/impulsive behavior, sleep disturbance, and suicidality.

Major Findings:

A negative correlation exists between CSF 5-HIAA and childhood problems associated with ADD/CD, aggression, depression and headaches. Lower CSF 5-

HIAA has now been found in DBD vs. OCD children. In late adolescents those with higher scores for aggressive/impulsive behaviors had greater mean scores on items related to family instability, lower levels of CSF and a greater incidence of suicide attempts compared to those with lower scores for aggressive/impulsive behavior. Disturbed family history per se was not related to levels of CSF 5-HIAA, possibly indicating various kinds of disturbed personality and behavior not of an aggressive and/or suicidal nature, which may not be associated with lower CSF 5HIAA. These findings support an inverse relationship between aggressive/impulsive behavior or behavior thought to indicate dyscontrol and disinhibition as trait characteristics and CSF 5-HIAA. Further, evidence seems to support impulsive/aggressive behavior being more clearly related to 5HT than a history of suicide attempts; suicidal behavior may be a form of aggressive behavior. Psychopathology in parents, particularly major affective disorder is associated with an increase in suicidal behavior in adolescent offspring vs. the offspring of control parents. There is some early indication that serotonergic traits, as described above, are more prevalent in the offspring of psychiatrically disturbed parents.

Significance to Mental Health Research:

CNS functioning has been less studied in some major groups of psychiatric patients; viz., personality disorders, alcoholics, and borderlines vs. affective and schizophrenic disorders. Studies of animal models, ADD/CD, and prisoners suggest a relationship between central neurotransmitter systems and aggressive behavior. These studies may contribute to identification of risk factors for anti-social, suicidal behaviors and alcohol-related behaviors and the possible alteration of those behaviors through neuropharmacological interventions.

Proposed Course of Project:

Preparation for the NIMH-NNMC collaborative project began in January 1973. The approval processes, both in terms of scientific merit and the protection of rights of patients, were completed in July 1974. Though active data collection of the NIMH-NNMC project is not now in process, this collaboration continues to be of mutual benefit to NIMH, NIAAA and NNMC. Collaboration continues also with NIMH (CPB and LDP): the project is proposed for three more years.

Publications:

Brown G, Linnoila M, Goodwin FK. Impulsivity, aggressivity and violence associated with suicidal behavior. In: Maris R, ed. Assessment and prediction of suicide. Guilford Press, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00287-01 LCS

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Stress Axis, Immune System-Derived Cytokines and Ethanol

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: R. Eskay Research Physiologist LCS, NIAAA

COOPERATING UNITS *(if any)*

Laboratory of Cell Biology, NIMH (L. Eiden, M. Palkovitz, E. Mezey);
Experimental Therapeutics Branch, NIMH (I. Mefford)

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- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Consumption of ethanol (Et) alters certain regulatory aspects of the hypothalamic-pituitary-adrenal axis (HPAA). Because the integrity of this system depends on the coordinated synthesis and secretion of specific regulatory substances at the hypothalamic (e.g., corticotropin-releasing hormone (CRH); vasopressin (AVP); biogenic amines), pituitary-gland (e.g., beta endorphin (BE); ACTH) and adrenal-gland (e.g., catecholamines; glucocorticoids) level, we have been evaluating the impact of Et at each level of the HPAA. Activation of the HPAA or hypercortisolism accompanies both short- and long-term consumption of Et and the Et withdrawal syndrome. Alcoholics often present with a pseudo-Cushing's syndrome in which some 17-40% of alcoholics do not respond to the dexamethasone suppression test during the first week of abstinence. Since a relative state of elevated glucocorticoids (chronic continuous or chronic intermittent) can lead to neural changes and even cell death, particularly in the hippocampus, the progressive loss of cognitive capacity in many alcoholics may indeed be due in part to hypercortisolemia and subsequent irreversible neural damage in the hippocampus and other areas of the central nervous system. Furthermore, armed with the concept of the bidirectional communication between the HPAA and the immune system. We are exploring whether or not certain immune system-derived cytokines may be ameliorating or accelerating neural death through endocrine or paracrine actions. Certainly cytokines stimulate diverse cell types in an attempt to repair cellular damage through intracellular signal amplification which could in concert with Et and glucocorticoids overstimulate selected neural populations leading to their demise.

Project Description:

Investigators:

R. Eskay	Physiologist	LCS, NIAAA
L. Eiden	Unit Chief	LCB, NIMH
I. Mefford	Special Expert	ETB, NIMH
E. Mezey	Guest Researcher	LCB, NIMH
M. Palkovitz	Visiting Scientist	LCB, NIMH

Objectives:

The ongoing aims of these studies are: 1) to profile ethanol-induced changes in the HPAA and immune system with a particular emphasis on the immune system derived cytokines which alter stress axis function, 2) to understand the sequence of events in these systems from membrane-receptor to intracellular-messenger systems to gene activity to physiological responses, 3) to determine the concentration-dependent and time-dependent effects of ethanol on the various cellular events as outlined in 1 and 2, and 4) to elucidate the consequences of ethanol's indirect effects on the HPAA and immune system, which may be mediated through elevated glucocorticoids and altered cytokine levels, respectively.

Methods Employed:

In an attempt to understand the precise mechanism or site of Et's activation of the HPAA, a series of studies were initiated in unrestrained, conscious adult male rats containing an indwelling jugular cannula for blood sampling and an indwelling intragastric cannula for ethanol administration. Animals received either a single infusion of a moderately high dose of ethanol (3.2gm/kg) or were continuously (0.5, 1, 3, 7 days) exposed to an ethanol/liquid diet via intragastric intubation in which blood ethanol levels were maintained between 150-300 mg% and compared to non-ethanol isocaloric control animals.

Major Findings:

Clearly, single-dose Et challenge of naive rats activates the HPAA, as indicated by the rapid elevation of plasma ACTH, BE, corticosterone (Cs), and catecholamine concentrations. Passive immunoneutralization of AVP or removal of the adrenal medulla prior to Et challenge revealed that neither adrenal medulla-derived epinephrine nor AVP was a significant regulator or coregulator of corticotrophin secretions following a moderately high, single-dose administration of Et.

When animals were continuously exposed (.5, 1, 3, 7 days) to Et/ liquid diet via intragastric intubation in which blood ethanol levels were maintained between 150-300 mg % and compared to non-Et isocaloric control animals, we observed interesting HPAA changes. In short, plasma levels of Cs remained elevated during the first 3 days. Consistent with a strong activation of the stress axis by Et, at least during the first day, paraventricular (PVN) CRH messenger (m) RNA was dramatically elevated following 24 hrs of Et exposure.

It returned to control levels by 3 days. In contrast to the observed CRH mRNA changes in the PVN, which is the primary source of CRH-containing cell bodies that project to the median eminence, CRH mRNA-containing neurons intrinsic to the olfactory bulb, cingulate, insular and parietal cortex regions demonstrated dramatically elevated CRH mRNA content only after 7 days of Et exposure. Although the functions of extrahypothalamic CRH neurons are not fully understood, this novel finding indicates that chronic Et exposure may enhance the functional activity of extra-neuroendocrine CRH neurons independent of the activation of the traditional stress axis.

Significance to Biomedical Research and the Program of the Institute:

The continued exploration of the effects of Et on fundamental physiological systems, such as the stress axis and immune system, should provide additional understanding of multiple, ethanol-induced pathological perturbations of cellular events that are associated with alcoholism. This, in turn, will hasten the development of effective therapeutic agents to treat patients with alcohol-related dysfunctions.

Proposed Course:

Although activation or disruption of the HPAA axis through Et abuse results in hypercortisolemia, just how this contributes to CNS dysfunction is not clear. Since the hippocampus appears to be particularly sensitive to one of the consequences of Et consumption, namely elevated glucocorticoids, we will profile the effects of Et exposure on hippocampal function. Furthermore, the likelihood that cytokines like IL-1 and TNF participate in normal physiological events such as modulation of the stress axis, in addition to their known roles in inflammation and host defence are being explored.

Publications:

Eskay RL, Grino M, Chen W. Interleukins, signal transduction, and the immune system-mediated stress response, Adv Exp Med Biol 1990;274:331-43.

Eskay RL, Eiden L, Mefford I. Immune system cytokines and stress-axis organ responses. In: Axelrod J, McCarty R, Kvetnansky R, eds. Stress: Neuroendocrine and Molecular Approaches. Gordon and Breach Publishers, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00277-03 LCS

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Non-human primate models of alcohol consumption and aggression

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. Higley	Senior Staff Fellow	LCS, NIAAA
Others:	B. Giblin	NRC Fellow	LCS, NIAAA
	D. Goldman	Section Chief	LCS, NIAAA
	M. Linnoila	Scientific Director	LCS, NIAAA
	D. Nielsen	Senior Staff Fellow	LCS, NIAAA

COOPERATING UNITS *(if any)*

Laboratory of Comparative Ethology, NICHD (S. Suomi, M. Champoux, K. Zajecik); Ebon (A. Dodson)

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3.4	1.4	2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

During the past year, research has been pursued concerning (1) Etiological mechanisms underlying individual differences in alcohol consumption in rhesus monkeys, with an emphasis on paternal genetic contributions; (2) The relationship between social competence and serotonin in ferally living monkeys: To assess the generalizability of our laboratory findings, and to obtain a subject pool of inappropriately aggressive individuals with sufficient numbers to perform parametric analyses, we continued to investigate ferally-living adolescent male monkeys drawn from a subject pool of 4500 rhesus monkeys living on an island off the coast of South Carolina; (3) Developmental patterns of rhesus monkeys selectively bred for extremes in CSF 5-HIAA: A major part of the past year has involved the development of a selective breeding program to breed for extremes in CSF 5-HIAA concentration. Both the parents and the infants have been carefully assessed to investigate parental genetic contributions to excessive aggression and alcohol consumption; (4) Genetic differences in the serotonin system using molecular genetics techniques, including amplification of the serotonin 1A receptor gene in monkeys with increased and diminished CSF 5-HIAA, development of fibroblast cell lines to acquire DNA, and the development of techniques to type tryptophan hydroxylase; (5) Assessment of the pharmacokinetics of imipramine in monkeys showing excessive anxiety under both baseline and stressful conditions.

Project Description:

Investigators:

J. Higley	Senior Staff Fellow	LCS, NIAAA
M. Champoux	Staff Fellow	LCE, NICHD
A. Dodson	Vet Technician	Ebon
B. Giblin	NRC Fellow	LCS, NIAAA
D. Goldman	Section Chief	LCS, NIAAA
M. Linnoila	Scientific Director	NIAAA
D. Nielsen	Senior Staff Fellow	LCS, NIAAA
S. Suomi	Chief	LCE, NICHD
K. Zajecik	Technician	LCE, NICHD

Objectives:

This project is concerned with the development of primate models of excessive alcohol consumption, anxiety, and excessive aggression. Principal research concerns the effect of developmental experience and genetic influences on alcohol consumption, anxiety, and aggression. This research is performed by tracing developmental pathways, effects of early rearing, and continuities of individual differences. Behavioral, hormonal, and neurotransmitter measurements, genetic probes, and psychopharmacological manipulations are its major tools.

Methods Employed:

Rhesus monkeys are studied longitudinally from birth through adulthood either in naturalistic settings or in well controlled laboratory settings to assess their potential for excessive alcohol consumption, anxiety, and aggression. Those reared in the laboratory are selectively bred for excessive alcohol consumption or CSF 5-HIAA concentration. They are then reared either as a control group in a setting approximating normative rhesus monkey social organization or in anxiety producing peer-only groups. As young adults, they are exposed to ethanol. Their rates of alcohol consumption are linked to their carefully controlled genetic and rearing backgrounds, and to psychobiological variables. In both the feral and laboratory environments, as the monkeys develop, their behaviors are systematically sampled across major developmental phases. Differences in anxiety, aggression, social competence, and alcohol consumption are linked to individual differences in CNS functioning using measurements of hypothalamic-adrenal-pituitary activity and neurotransmitter system activity as assessed via CSF monoamine concentrations.

Major Findings:

1. Etiological mechanisms underlying individual differences in alcohol consumption in rhesus monkeys, with an emphasis on paternal genetic contributions:

In previous studies of rhesus monkeys, we found strong environmental influences on alcohol consumption with rearing conditions that increase anxiety and fearfulness producing excessive alcohol consumption. However, independent of rearing there are widespread interindividual differences, and we hypothesized that these individual differences might be genetically mediated. During the past year we have assessed 20 paternal half-siblings to investigate genetic contributions to alcohol consumption. We are now approaching a sufficient sample size to allow an analysis of the genetic contribution to alcohol consumption in rhesus monkeys. Analyses of these paternal half-siblings show potentially strong paternal heritable influences on alcohol consumption.

2. The relationship between social competence and serotonin in ferally living monkeys:

Given the findings in humans of an association between decreased 5-HIAA and aggression, and increased norepinephrine and timidity, and our findings reported last year showing similar parallels in rhesus monkeys, an extensive investigation of ferally living rhesus monkeys on an island in South Carolina was continued. Because highly aggressive monkeys are difficult to find in the laboratory, this project has been crucial to identify highly aggressive and submissive monkeys from which to obtain CSF and blood plasma samples. During the past year, we have increased our sample size to nearly 80 subjects, obtained repeated CSF samples from approximately 50 subjects, and placed radiocollars on 25 of the subjects in order to collect behavioral data. A wide variety of subjects have been assessed. Monkeys who had the highest plasma concentrations of ACTH in the first year were less likely to be captured in the past year. Monkeys who remained in their natal troops showed stable interindividual differences in CSF monoamine concentrations. A major contribution from this study was the acquisition of a breeding male with the lowest CSF 5-HIAA that we have sampled. He was expelled from the island for excessive aggression and is now being used to selectively breed with females having diminished CSF 5-HIAA to produce infants with low CSF 5-HIAA. This year will be a crucial year to the project as most of the males will move from their natal troop to new troops which will allow us to link their integration into the new troops and survival rates with previous measures of social competence and interindividual differences in aggression. We plan to correlate these measures of social competence and adjustment to their new social environment with the CSF and plasma variables we have quantified.

3. Developmental patterns and predictors of rhesus monkeys selectively bred for extremes in CSF 5-HIAA:

Given the genetic effects on alcohol consumption in humans, and the findings of genetic effects on 5-HIAA, a major part of the past year has involved the development of a selective breeding program to breed for extremes in CSF 5-HIAA concentration. To date 8 infants have been born. Infants born to these parents have been removed from their mothers at birth and placed in the neonatal nursery to carefully control their rearing, and to remove parental environmental influences. Both the parents and the infants have been carefully assessed to investigate parental genetic contributions to excessive aggression and alcohol consumption. The parents were assessed prior to and during pregnancy for levels of aggression and concentrations of CSF monoamines and plasma hormone concentrations. The infants were assessed using a battery of variables including neonatal temperament ratings, sleep and state measures, monthly CSF monoamine and plasma hormone samples, and systematic samples of social behavior. We will carefully follow these individuals as they develop.

4. Genetic differences in the serotonin system using molecular genetics techniques:

In collaboration with Dr. Goldman's group, two studies were initiated to assess etiological mechanisms producing genetic differences in CSF 5-HIAA. Dr. Giblin has initiated work on the amplification of the serotonin 1A receptor gene in monkeys specifically selected because of their extremes in CSF 5-HIAA. To date the technique has worked well with rhesus monkeys allowing assessment of 6 subjects. We have also begun to develop fibroblast cell lines to acquire DNA for subsequent assessments. Under the direction of Dr. Nielsen, a planned initiative for the next year will be to use the techniques that he has developed in two other species to type the gene for tryptophan hydroxylase in rhesus monkeys.

5. Assessment of the pharmacokinetics of imipramine in monkeys showing excessive anxiety under both baseline and stressful conditions:

Preliminary data indicated that imipramine metabolism varies according to the degree of stress a subject is undergoing. As part of an ongoing investigation of this phenomenon, we administered imipramine to 8 adolescent rhesus monkeys both during home-cage baseline conditions and during the stress of social separation. Blood imipramine samples are yet to be assayed for this project; however, consistent with an earlier study, levels of aggression decreased and play levels increased.

Significance to Biomedical Research and the Program of the Institute:

Our discovery of a primate model of excessive alcohol consumption allows the development of etiological models and experimental treatments of human alcoholism. We have begun to test specific hypotheses concerning the etiology of human alcoholism and its treatment. In terms of biological links to aggression, given the problems in studying aggression in humans, nonhuman primates are ideal candidates to test the correlations between serotonin and aggression and to provide treatments for low serotonin mediated aggression. In addition, as this nonhuman primate model is adopted by others, researchers will be able to carefully control rearing histories, data collection, and test psychobiological mechanisms in a manner which is impossible in humans.

Publications:

Higley JD, Suomi SJ, Linnoila M. Parallels in aggression and serotonin: Development, rearing history, and sex differences. In: Van Praag HM, Plutchik R, Apter, A, eds. Violence and Suicidality: Perspectives in Clinical and Psychobiological Research. Clinical & Experimental Psychiatry, Monograph No. 3. New York: Brunner/Mazel Publishers, 1990;245-56.

Higley JD, Suomi SJ, Linnoila M. Serotonin in nonhuman primates: gender, gearing, and developmental correlates with behavioral timidity and affective psychopathology. In: Coccaro EF, Murphy DL, eds. Serotonin in Major Psychiatric Disorders: APA Press Progress in Psychiatry Monograph Series. Washington, DC: American Psychiatric Press, Inc., 1990;27-46.

Higley JD, Suomi SJ, Linnoila M. CSF Monoamine Metabolite Concentrations vary according to age, rearing, and sex, and are influenced by the stressor of social separation in rhesus monkeys, Psychopharmacol 1991;103:551-6.

Higley JD, Hopkins WD, Thompson WW, Byrne EA, Hirsch RM, Suomi SJ. Peers as primary attachment sources in yearling rhesus monkeys (Macaca mulatta), Developmental Psychology, in press.

Higley JD, Hasert MF, Suomi SJ, Linnoila M. A new nonhuman primate model of alcohol abuse: effects of early experience, personality, and stress on alcohol consumption, Proceedings of the National Academy of Science USA, in press.

Suomi SJ, Higley JD. Rationale and methodologies for developing nonhuman primate models of prenatal drug exposure. In: Methodological Issues in Controlled Studies on Prenatal Effects of Drugs of Abuse: National Institute of Drug Abuse Research Monograph, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AA 00238-09 LCS

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
CSF Neuropeptides in Alcohol Withdrawal and Brain Disease

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	M. Linnoila	Scientific Director	NIAAA

COOPERATING UNITS *(if any)*
VA Medical Center, Washington, D.C. (J. Hawley); Department of Psychiatry, Emory University, Atlanta, GA (C. Nemeroff, G. Bissette).

LAB/BRANCH
Laboratory of Clinical Studies

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.2	1.0	0.2

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
  ☐ (a1) Minors
  ☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Severity of withdrawal symptoms from alcohol was quantified in alcoholics admitted to the Neurology Ward of the Washington, DC VA Hospital. Cerebrospinal fluid (CSF) samples were repeatedly obtained early during withdrawal after all symptoms had subsided. Concentrations of the monoamine neurotransmitter norepinephrine and its major metabolite MHPG were measured at NIH. Significant positive correlations were observed between indices of elevated norepinephrine turnover and several signs of alcohol withdrawal. We are currently measuring peptides known to be released in synapses simultaneously with norepinephrine. We are continuing to increase our sample size.

Previous title: CSF Neuropeptides and Prostaglandins in Alcohol Withdrawal and Brain Disease

Project Description:

Investigators:

M. Linnoila	Scientific Director	NIAAA
G. Bissette	Assoc. Prof. Psychiatry	Emory University Atlanta, GA
J. Hawley	Neurologist	VA Medical Center Washington, DC
C. Nemeroff	Chairman of Psychiatry	Emory University Atlanta, GA

Objectives:

Many symptoms of alcohol withdrawal are indicative of sympathetic nervous system overactivity (excitability, tremor, sweating, etc.) The activity of the sympathetic nervous system is partially controlled by central nervous circuits, which use norepinephrine as a transmitter. We are investigating activity in alcoholics undergoing severe withdrawal, and correlating the degree of clinical symptoms with biochemical changes indicative of the amount of norepinephrine released from presynaptic nerve terminals. Furthermore, we are investigating the regulation of the noradrenergic neurone systems.

Methods Employed:

Clinical symptoms of withdrawal are rated by Dr. James Hawley in the Neurology Department, at the Veterans Administration Hospital in Washington, D.C. where the patients are treated and studied under a VA approved protocol. The instrument used to quantify severity of individual withdrawal symptoms, as well as the total score, is the extensively validated Gross rating scale. Dr. Hawley also performs two LPs on every subject, one early and the other late during withdrawal. CSF will be assayed by the Laboratory of Clinical Studies, NIAAA with liquid chromatography using electrochemical detection. Neuropeptides are measured with radioimmunoassays.

Major Findings:

CSF norepinephrine and MHPG concentrations were significantly ($p<.01$) higher in the samples obtained from alcoholics early as compared to late withdrawals. CSF norepinephrine and MHPG concentrations correlated highly with each other and with the severity of sweating, anxiety, tremor, heart rate and blood pressure in the withdrawing alcoholic patients.

Significance to Biomedical Research and the Program of the Institute:

These results provide the strongest available evidence of significant central noradrenergic overactivity during alcohol withdrawal in humans. Furthermore, the significant associations between the biochemical results and clinical symptoms are suggestive of the role noradrenergic overactivity plays as a possible cause of these symptoms.

Proposed Course:

We are collecting cerebrospinal fluid, urine, and blood samples in patients with alcohol-induced chronic brain disease and in a new group of patients undergoing withdrawal from alcohol to investigate possible monoaminergic deficits. Such deficits, if found, will provide new rationales for treatment strategies. Furthermore, we are quantifying neuropeptides known to be associated with the presynaptic release of norepinephrine. Such measurements are expected to elucidate mechanisms involved in the dysregulation of the noradrenergic systems during withdrawal from alcohol and in alcohol-induced chronic organic brain syndromes.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AA 00258-07 LCS

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Violent Behavior, Neurotransmitters, Glucose Metabolism and Alcohol Abuse

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	M. Linnoila	Scientific Director	NIAAA
Other:	D. Goldman	Section Chief	LCS, NIAAA

COOPERATING UNITS *(if any)*
Department of Psychiatry, University of Helsinki (M. Virkkunen)

LAB/BRANCH
Laboratory of Clinical Studies

SECTION
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NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.4	0.2	0.2

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☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
  ☐ (a1) Minors
  ☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have investigated neurotransmitter metabolites and glucose metabolism in, incarcerated violent offenders, arsonists and healthy volunteers. We have found that low cerebrospinal fluid (CSF), 5-hydroxyindoleacetic acid (5HIAA) concentrations and hypoglycemia during oral glucose tolerance tests are associated with each other and impulsive violent acts and fire setting. In a follow-up study, we found that a low blood glucose nadir and low CSF 5HIAA concentration were powerful predictors of recidivism among impulsive violent offenders and fire setters. We are currently collecting lymphocytes for molecular genetic studies from violent offenders, their family members and appropriate controls.

Project Description

Investigators:

M. Linnoila	Scientific Director	DICBR, NIAAA
D. Goldman	Section Chief	LCS, NIAAA
M. Virkkunen	Senior Lecturer	Univ. of Helsinki, Finland

Objectives:

To investigate psychobiological and genetic variables associated with impulsive and violent behaviors as well as alcohol abuse in humans.

Methods Employed:

Cerebrospinal fluid neurotransmitters and neurotransmitter metabolites have been quantified in samples obtained from violent offenders, arsonists and healthy volunteers. The subjects have been administered oral glucose tolerance tests and MMPI's, and they have undergone careful forensic psychiatric examinations. A follow-up study on recidivism has been completed using the criminal register of Finland to detect repeat crimes. Lymphocytes are currently being collected from violent offenders and their families to be used for genetic linkage studies.

Major Findings:

Compared to healthy volunteers, violent offenders have low cerebrospinal fluid 5HIAA and 3-methoxy-4-hydrophenyl glycol concentrations. Eleven of twenty arsonists became hypoglycemic. Psychobiological variables classified correctly 84% of subjects into the recidivist and nonrecidivist categories.

Significance to Biomedical Research and the Program of the Institute:

Alcohol abuse is associated with large proportion of violent offenses and arsons. It has also been associated with low cerebrospinal 5HIAA concentration. We have demonstrated clear associations between low cerebrospinal fluid 5HIAA concentration, alcohol abuse and violent behavior. Furthermore, we have found in arsonists, associations between low glucose metabolism and alcohol abuse. These findings, if replicated by others, can form a rational basis for treatment interventions in these heretofore difficult-to-treat individuals.

Proposed Course:

We are collecting lymphocytes in a new sample of subjects to relate the described findings to possible Y-chromosome abnormalities. We will enlarge our sample to blood relatives of the index individuals and appropriate controls to investigate the heredity of these conditions. We have underway a study on circadian rhythms in violent offenders because of their frequent complaint of insomnia.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00272-04 LCS

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

CSF Monomamine Metabolites in Alcoholic Patients Who Attempt Suicide

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Pnncipal Investigator.) (Neme, title, laboratory, and institute effiliation)*

PI:	M. Linnoila	Scientific Director	NIAAA
Others:	D. George	Acting Section Chief	LCS, NIAAA
	J. DeJong	Staff Fellow	LCS, NIAAA
	D. Lamparski	Guest Researcher	LCS, NIAAA

COOPERATING UNITS *(if eny)*

None

LAB/BRANCH

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NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2	2	

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☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Reduced cerebrospinal fluid levels of the serotonin metabolite 5-hydroxyindoleacetic acid have been reported to be commonly associated with suicidal behavior. Alcoholics are known to often manifest suicidal behavior. Therefore, we compared cerebrospinal fluid levels of monoamine metabolites among alcoholics who had (N=18) or had not (N=132 attempted suicide, and controls (N=29). There were no significant differences among the three groups for cerebrospinal fluid concentrations of either 5-hydroxyindoleacetic acid, the dopamine metabolite homovanillic acid, norepinephrine, or the norepinephrine metabolite 3-methoxy-4-hydroxypenylglycol. However, in an expanded data set of almost 300 alcoholics there were significant differences for age of onset alcoholics who attempted suicide had an early age of onset of heavy drinking. They also had significantly more lifetime psychiatric diagnoses of major depression, antisocial personality disorder, panic, phobic disorder and more family history of alcoholism.

This project has been terminated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00288-01 LCS

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT (*80 characters or less. Title must fit on one line between the borders.*)

CNS Glutamate and c-fos mRNA in Alcohol Withdrawal

PRINCIPAL INVESTIGATOR (*List other professional personnel below the Principal Investigator.*) (*Name, title, laboratory, and institute affiliation*)

PI:	P. Morgan	Visiting Associate	LCS, NIAAA
Others:	M. Linnoila	Scientific Director	NIAAA
	J. Karanian	Research Physiologist	LCS, NIAAA
	K. Wozniak	Visiting Associate	LCS, NIAAA

COOPERATING UNITS (*if any*)

Laboratory of Neurophysiology, NINDS (S. Nadi)

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

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NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.4	1.4	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (*Use standard unreduced type. Do not exceed the space provided.*)

Activation of brain cells by receptor activation or changes in ion flux can result in the rapid and transient expression of certain key genes, such as the c-fos gene, which are capable of controlling and orchestrating the transcription of a large number of dependent genes. Such altered gene expression may underlie normal, as well as pathophysiological, long term changes in cell function.

We have mapped the expression of the c-fos gene in rat brain during alcohol intoxication and withdrawal and have identified several brain structures in which expression is increased during alcohol withdrawal. We have demonstrated that the regional expression of c-fos mRNA observed during alcohol withdrawal can be mimicked by acute administration of N-methyl-D-aspartic acid (NMDA) and stereospecifically blocked by the NMDA receptor/ionophore blocker, MK-801. We have also demonstrated that levels of glutamate (an endogenous ligand for NMDA receptors), but not other excitatory and inhibitory amino acid putative neurotransmitters, are increased in the brain during alcohol withdrawal.

Current research is focused on pharmacologically characterizing the increased expression of c-fos mRNA observed during alcohol withdrawal. In addition we are developing and screening DNA probes for other second messenger-dependent genes which may exhibit altered expression during alcohol intoxication or withdrawal.

Project Description:

Investigators:

P. Morgan	Visiting Associate	LCS, NIAAA
J. Karanian	Research Physiologist	LCS, NIAAA
M. Linnoila	Scientific Director	NIAAA
S. Nadi	Special Expert	LN, NINDS
K. Wozniak	Visiting Associate	LCS, NIAAA

Objectives:

The prime objective of these studies is to elucidate clinically relevant molecular changes in the brain which result from alcohol exposure and withdrawal. We have focused attention on the expression of Immediate-Early response genes, such as the protooncogene c-fos, in response to alcohol exposure and withdrawal. Expression of Immediate-Early response genes is a useful 'marker' of cellular activity, since these genes are rapidly and transiently expressed in response to cell stimulation or second messenger activation (eg. calcium, cyclic adenosine monophosphate, and phosphatidyl inositol) in many, if not all, excitable cells. A major objective of current studies is to pharmacologically characterize the altered regulation of gene expression observed during alcohol withdrawal.

Methods Employed:

Automatic vapor inhalation chambers are employed to expose Sprague Dawley rats to a constant concentration of alcohol vapor. Blood levels of alcohol and brain levels of mRNA, drug receptors, and endogenous amino acids are determined at different stages of alcohol intoxication or withdrawal, in the presence or absence of acute drug treatments, using the following methods: 1) Blood alcohol levels are determined by standard enzyme (alcohol dehydrogenase) assay. 2) Specific mRNA species are extracted from brain tissue by conventional separation technologies involving ultracentrifugation and agarose gel electrophoresis. 3) Radioactive (32P and 35S) DNA probes are synthesized and hybridized to select mRNA species by Northern blot techniques. 4) The regional distribution of specific mRNAs in the brain are mapped by quantitative in situ hybridization. 5) Affinity and density of drug receptors are estimated by ligand binding using appropriate tritiated ligands. 6) Endogenous levels of amino acids in the brain are estimated using microdialysis sampling and HPLC separation and electrochemical detection.

Major Findings:

Expression of the Immediate-Early response gene c-fos is significantly ($p<0.01$) increased during alcohol withdrawal, but not during intoxication. Peak expression is observed at 8 hours following the initiation of withdrawal. During peak expression the distribution of c-fos mRNA in brain is highly discrete: expression is increased several fold in the dentate gyrus of the hippocampus ($p<0.01$) and the piriform cortex ($p<0.01$) whereas expression in other brain areas is not significantly increased. The regional distribution of c-fos mRNA observed during alcohol withdrawal is mimicked by acute administration of N-methyl-D-aspartic acid (NMDA). The NMDA receptor/ionophore blocker, MK-801, stereospecifically blocks NMDA-induced expression of c-fos mRNA ($p<0.01$); it also blocks the increased expression of c-fos mRNA observed during alcohol withdrawal ($p<0.01$). Brain levels of glutamate (the endogenous ligand for the NMDA receptor), sampled from the striatum by in vivo microdialysis, were significantly higher in alcohol withdrawing animals compared to controls ($p<0.01$). Concomitant determinations of other endogenous putative neurotransmitter amino acids including aspartate, gamma amino butyric acid (GABA), and taurine were at levels not significantly different from control.

Significance to Biomedical Research and the Program of the Institute:

These findings strongly suggest that during alcohol withdrawal neurotransmission at NMDA receptors is increased. It also strongly suggests that brain structures such as the dentate gyrus of the hippocampus and the piriform cortex (which are known to transmit and receive information via afferent and efferent glutaminergic pathways) are particularly activated during alcohol withdrawal. The ability to map Immediate-Early response gene expression now enables us to visualize and quantify nucleotide changes occurring in the brain as a result of activity during alcohol withdrawal. This molecular model can now be employed to: (a) Assess strategies directed at reducing the molecular changes occurring during alcohol withdrawal; and (b) Further understand the role of neurotransmitter pathways in alcohol withdrawal and thereby identify likely therapeutic agents and strategies which may be effective in counteracting pathophysiological changes which occur during alcohol withdrawal.

Proposed Course:

We are currently investigating the effects of non-glutaminergic compounds on the expression of Immediate-Early response genes. We are presently focusing on benzodiazepines and related compounds, some of which have been demonstrated to be of therapeutic benefit during alcohol withdrawal. We are also developing and screening probes for other genes which may exhibit enhanced or reduced expression during alcohol intoxication and withdrawal. The c-fos gene and many of the Immediate-Early response genes are expressed in response to changes in all known major second messenger systems. We are concentrating efforts on developing probes for genes which may be expressed by, and only by, an increase in activity of a single second messenger. We are currently developing probes for mRNA coding for substrates of protein kinase C and for genes coding for proteins with binding affinity to the cyclase response element (CRE, a trans-activating enhancer sequence similar to the cognate sequence to Fos, which may be unique to the cAMP/A-kinase second messenger system). Quantitative brain microdialysis studies are also in progress to estimate the concentration of endogenous compounds which may account for the altered gene expression observed during alcohol withdrawal.

Publications:

Morgan PF, Linnoila M. Regional induction of c-fos mRNA by NMDA: a quantitative in situ hybridization study, Neuroreport 1991;2:251-4.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AA 00289-01 LCS

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
The Effects of Ethanol on Central Dopaminergic Function

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	K. Wozniak	Visiting Associate	LCS, NIAAA
Others:	M. Linnoila	Scientific Director	NIAAA

COOPERATING UNITS (if any)
Biological Psychiatry Branch, NIMH (A. Pert, A. Mele).

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NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

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☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Dopamine has been implicated as one of the primary neurotransmitters responsible for mediating the reinforcing properties of various substances of abuse, including ethanol. We have investigated the effect of local perfusion of ethanol into certain brain areas on extracellular dopamine, and its metabolites DOPAC (di-hydroxyphenylacetic acid) and HVA (homovanillic acid). We have demonstrated that ethanol increases extracellular dopamine concentrations in a dose-related fashion. This increase in dopamine was dependent on normal calcium channel function and occurred at brain concentrations relevant to intoxication in humans (<100mM). We have also shown that the different alcohols exhibit a structure-activity relationship with regard to their ability to increase dopamine with butanol> ethanol> methanol. Pretreatment with antagonists at 5-HT3 receptors effectively attenuated the ethanol-induced increase in dopamine levels. Current research is focussed on further elucidating the nature of this dopamine increase. In addition, we are developing studies to investigate the effects of ethanol on dopamine function in chronically ethanol treated animals.

Project Description:

Investigators:

K. Wozniak	Visiting Associate	LCS, NIAAA
M. Linnoila	Scientific Director	NIAAA
A. Mele	Visiting Fellow	BPB, NIMH
A. Pert	Section Chief	BPB, NIMH

Objectives:

The prime objectives of these studies are to elucidate physiologically relevant changes in central neurotransmitter function following ethanol administration. Since dopamine is thought to be at least partially responsible for mediating the euphorigenic and reinforcing properties of various substances of abuse, special attention has been focused on changes in dopaminergic function. Additionally, since recent evidence suggests a modulatory role for 5-HT3 receptors in dopamine function, the effects of pretreatment with 5-HT3 receptor antagonists on ethanol-induced changes in dopamine were investigated.

Methods employed:

1. Stereotaxic implantation of microdialysis probes into specific brain regions, including the nucleus accumbens and substantia nigra.
2. Microdialysis of specific structures with focally applied ethanol, and other alcohols.
3. Estimation of endogenous dopamine levels, and that of the dopamine metabolites dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), using HPLC separation with coulometric and/or electrochemical detection.

Major findings:

Extracellular dopamine levels were significantly increased by focal perfusion with various concentrations of ethanol, in both the nucleus accumbens and the substantia nigra. The different alcohols demonstrated a structure-activity relationship with regard to their potency in affecting dopamine concentrations; with the four carbon atom alcohol butanol, being more potent then the two carbon containing alcohol ethanol, which in turn was more potent than the single carbon containing alcohol, methanol. Furthermore, the ethanol-induced increase in dopamine was attenuated in the presence of calcium channel blockade, suggesting that it was physiologically mediated, as a result of normal exocytotic processes. Finally, pretreatment of rats with an antagonist at 5-HT3 receptors effectively attenuated the ability of ethanol to increase extracellular dopamine. This is evidence for a modulatory role of 5-HT3 receptors in the effects of ethanol on dopamine function.

Significance to Biomedical Research and the Program of the Institute:

These findings support the notion that substances of abuse act to increase extracellular dopamine, as one of their major actions. Additionally, this ethanol-induced increase in dopamine seemed to represent a physiologically relevant response, since it was dependant on normal calcium channel function. Furthermore, the finding that alcohols exhibited potencies with regard to their ability to elevate extracellular dopamine, similar to their potency with regard to other properties such as intoxication and antagonism of NMDA (N-methyl-D-aspartic acid), suggests that this change in dopamine may be relevant to the pharmacology of alcohols. The ability of pretreatment with 5-HT3 antagonists to attenuate the effects of ethanol on dopamine suggests a modulatory role for this receptor in ethanol's actions. Furthermore, this implicates a possible use for these agents in the abuse of alcohol and other substances.

Proposed course:

We are currently investigating the effects of ethanol on dopaminergic function further. This includes investigating the nature of the response to ethanol in chronically treated animals, to elucidate any possible changes in dopaminergic function underlying the phenomena of tolerance and withdrawal following ethanol exposure. Furthermore, we are also attempting to further clarify the exact nature of the dopamine response to ethanol, by means of the potent toxin, tetrodotoxin. Other 5-HT3 antagonists demonstrating stereospecificity in their affinity for the 5-HT3 sites, will be utilized in an attempt to antagonize the ethanol response, in an effort to define the specificity of this effect to the 5-HT3 receptor site.

Publications:

Wozniak KM, Pert A, Linnoila M. Antagonism of 5-HT3 receptors attenuates the effects of ethanol on extracellular dopamine, Eur J Pharmacol 1990;187:287-9.

Wozniak KM, Pert A, Mele A, Linnoila M. Focal application of alcohols elevates extracellular dopamine in rat brain: a microdialysis study, Brain Res 1991;540:31-40.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00231-09 LCS

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Central and Peripheral Nervous System Function in Abstinent Alcoholics

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	M. Eckardt	Section Chief	LCS, NIAAA
Others:	D. Flowers	Special Volunteer	LCS, NIAAA
	D. George	Acting Section Chief	LCS, NIAAA
	M. Linnoila	Scientific Director	NIAAA

COOPERATING UNITS *(if any)*
Washington University (J. Rohrbaugh); Vanderbilt University (P. Martin); VAMC, Charleston, SC (B. Adinoff); Clinical Neuroendocrinology Branch, NIMH (P. Gold); Laboratory of Personality and Cognition, NIA (H. Weingartner).

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SECTION
Section of Clinical Science

INSTITUTE AND LOCATION
NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

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☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Behavioral deficits in alcoholics have been conceptualized in terms of two neuropathologically distinct syndromes: alcoholic dementia and Korsakoff's psychosis (alcohol amnestic disorder). Alcoholic dementia is characterized by diffuse cortical damage primarily related to the neurotoxicity of alcohol; Korsakoff's psychosis is associated with subcortical lesions due to nutritional (thiamine) deficiency. Severe memory impairment with relative sparing of other intellectual functions distinguishes Korsakoff's psychosis from alcoholic dementia (which may be clinically indistinguishable from the most common cause of dementia, Alzheimer's disease). We have recently found that sleep in Korsakoff patients is characterized by a reduced REM latency compared to normal volunteers, whereas Alzheimer patients have normal latencies. Furthermore, delta sleep is reduced in Alzheimer's disease, but is normal in Korsakoff's psychosis. Most patients with demonstrated reduced daily excretion of the major urinary metabolite of melatonin, hydroxmelatonin, present with Korsakoff's psychosis. This finding is suggestive of impaired pineal function. Genetic differences in thiamine metabolism may predispose patients to develop Korsakoff's psychosis. Most patients with Korsakoff's psychosis whom we have studied have had a transketolase with reduced affinity for thiamine pyrophosphate. Modifying activation and arousal by pharmacologic modulation of neurotransmitter systems may be effective in treatment of Korsakoff's psychosis, whereas alcoholic dementia may require treatment strategies similar to those in Alzheimer's disease. This protocol is intended to utilize clinical, neuroradiological, physiological, and neuropharmacological studies to differentiate these two pathologic entities, to follow a longitudinal course, and to relate variables in treatment protocols to outcome.

Project Description

Investigators:

M. Eckardt	Section Chief	LCS, NIAAA
B. Adinoff	Chief	VAMC, Charleston, SC
D. Flowers	Special Volunteer	LCS, NIAAA
T. George	Acting Section Chief	LCS, NIAAA
P. Gold	Chief	CNE, NIMH
M. Linnoila	Scientific Director	NIAAA
P. Martin	Associate Professor	Vanderbilt Univ. Nashville, TN
J. Rohrbaugh	Associate Professor	Washington Univ., St. Louis, MO
H. Weingartner	Section Chief	LPC, NIA

Objectives:

Chronic organic brain syndromes due to alcoholism constitute the second most common cause of dementia in adults (approximately 10 percent), ranking next to senile dementia of the Alzheimer's type (40-60 percent). Currently, a large proportion of dementing illness can be diagnosed with certainty only by examining the microscopic structure of the brain at autopsy. The cross sectional clinical picture of alcohol-related cognitive decline may be difficult to distinguish from that of the more prevalent primary degenerative dementia (Alzheimer's disease). Chronic alcohol abuse may lead to two clinically and neuropathologically distinguishable syndromes: alcoholic dementia and alcohol amnestic syndrome (also called Korsakoff's psychosis). These two organic brain syndromes may represent extremes of the spectrum of cognitive impairments related to chronic alcoholism. Alcoholic dementia is characterized by global intellectual decline, whereas the salient clinical feature of the alcohol amnestic syndrome is a severe and persistent memory deficit with relative sparing of other intellectual functions. The majority of alcoholic patients have aspects of both syndromes; presumably the midline subcortical lesions due to thiamine deficiency may explain the amnestic component, where the diffuse bilateral cortical damage resulting from alcohol neurotoxicity explains the global cognitive loss. It has been postulated that polymorphisms of thiamine-requiring enzymes may influence which clinical syndrome predominates. Most of the patients with Korsakoff's psychosis in whom we have studied fibroblast transketolase have had an elevated K_M for thiamine pyrophosphate in comparison with the K_M derived from normal controls. We have found that patients with relatively "pure" amnestic characteristics have demonstrated episodic memory impairments that resemble those found in depression and Parkinson's disease and are distinguishable from the semantic or knowledge memory deficits found in Alzheimer's disease. Furthermore, we have demonstrated significant differences in the pattern of sleep EEG abnormalities in Korsakoff's psychosis patients compared with those with Alzheimer's disease. The sleep of Korsakoff patients resembles that of patients with depression (increase arousal and shortened REM latency). We postulate that treatment strategies directed toward modifying activation and arousal by pharmacologic modulation of neurotransmitter systems may be effective in treatment of the alcoholic amnestic syndrome. This situation is analogous to the benefits derived form pharmacotherapy on depression and Parkinson's disease, whereas alcoholic dementia requires treatment approaches similar to those in Alzheimer's disease.

Methods Employed:

We will study two groups of controls (healthy nonalcoholics and alcoholics abstinent for at least six months) and four groups of patients (detoxified alcoholics who have been abstinent from alcohol for at least one week; alcoholics withdrawn from alcohol who have been abstinent for at least three weeks; alcohol amnestic patients; and alcoholic dementia patients), using the following clinical, physiological and neurochemical tests: 1) neuropsychological evaluation of patients to determine whether they are predominantly amnestic or demented (in collaboration with Dr. Weingartner); 2) norepinephrine response to orthostasis; 3) dose-response to norepinephrine infusion 4) norepinephrine and endocrine responses to insulin tolerate test; 5) catecholamine and neuropeptide metabolism in cerebrospinal fluid versus plasma and urine; 6) vasopressin response to hypertonic saline infusion; 7) thyrotropin-releasing hormone and gonadotropin-releasing hormone stimulation tests (in collaboration with Dr. Gold); 8) circadian rhythms of melatonin, body temperature, and activity; and 9) sleep EEG.

Major Findings:

A number of potentially important findings have emerged. These are as follows: 1) In the saline infusion (vasopressin test) it appears that middle-aged alcoholics have blunted responses of vasopressin to the saline infusion. In other words, the normal age-related increase in vasopressin response to saline is considerable less in alcoholics. This would suggest damage to central hypothalamic mechanism, possibly as a result of drinking, since the young alcoholics do not show a similar pattern. To be sure of this, we have to increase our number of middle-aged controls (40-60 years) as we are presently doing. 2) The norepinephrine infusion data have been analyzed. The previously reported blunting of response to the low rate of norepinephrine infusion was confirmed. Since plasma norepinephrine concentrations do no differ in this duty between those two groups, we conclude there is a subsensitivity in the alcoholics. However, the importance of this is hard to determine since, with the higher rates of infusion, alcoholics behave much as controls. Analysis of heart changes during the norepinephrine infusion suggests that central baroreceptor mechanism are not altered in young alcoholics. 3) The insulin challenge test has been pursed and we have now demonstrated that amongst the alcoholic population there are two groups. One shows enhanced responsiveness to insulin, i.e. increased medullary responses, while the other shows a blunting of this response. We are presently studying other subjects to see why such a dichotomy might exist. Analysis of the pituitary hormone response to insulin-induced hypoglycemic is still pending. 4) Analysis of the pituitary response to the TRH/LHRH infusion has suggested that a number of alcoholics may be borderline hypothyroid. Another batch of samples has been sent to the laboratory and these will be analyzed subsequently. 5) The CRH challenge showed clearly that in alcoholics given CRH one week after withdrawal, the majority had markedly blunted ACTH responses. This may reflect adrenal hyperplasia in alcoholics, possibly secondary to the effects of withdrawal. In those subjects in which testing was performed after a long period of sobriety (up to several years in one patient), a consistent increase in ACTH responses to CRH was observed. This suggest that alcohol has a profound attenuating effect on hypothalamic responsiveness to CRH and that many years may be necessary before full return to normal function.

Significance to Biomedical Research and the Program of the Institute:

Chronic organic brain syndromes due to alcoholism are responsible for approximately 10 percent of dementia in the adult population. The fact that only a small population of alcoholic develop complication of alcoholism suggest the importance of predisposing factors. We will attempt to identify genetic factors that may be predictive of which individuals will develop the alcohol amnestic syndromes if they abuse alcohol. We plan to develop a clinical, physiological, and biomedical classification system of alcohol-associated chronic organic brain syndromes; this system will have diagnostic, prognostic, and therapeutic applications.

The findings of abnormal peripheral sympathetic responsiveness, especially that of the adrenal gland in young alcoholics may help us understand the damage produced by alcohol. However, they may also reflect a trait predisposition in alcoholics which could conceivably be a biological marker for the risk of alcoholism and may throw some light on the etiology of alcoholism. Clearly, we need to study nonalcoholic adult children of alcoholics to determine whether similar abnormalities exist, and thus to establish or refute whether this represents an inherited contribution to the disease. Further findings suggest that in most recently abstinent alcoholics the total ACTH response to CRH stimulation is decreased, and that this response tends to "normalize" over time. Changes int he correlation between baseline cortisol levels and HPS axis responses to CRH were evident even in alcoholics with greater than six months abstinence. This suggests that alternations in HPA axis functioning may persist for prolonged periods following cessation of drinking. It is not clear from this study if these findings are due to chronic effects of ethanol or to effects of subsequent withdrawal. syndrome.

Although elevated baseline-free cortisol levels were not observed in any of the alcoholic groups studied, cortisol response to CRH stimulation was proportionately greater in the one week- and three week-abstinent low ACTH responders compared to controls. This observation is compatible with the development of hyperplasia and hyperresponsiveness of the adrenal cortex, which is known to occur after even a few days of stimulation with exogenous ACT\H or with the application of experimentally induced stress. As the group of alcoholics we tested at one week- and three week-abstinence had experienced no more than mild withdrawal symptoms, were in relatively good health, and were not tested until one week following their last drink, the findings reported are most likely quite subtle compared to the changes one might observe in a poorly nourished alcoholic following delirium tremens. Our observations may, therefore, suggest the pituitary responsiveness may be severely impaired for a prolonged period of time following abstinence in more severely impaired alcoholics, and could contribute to the medial and psychological problems seen in this group.

Proposed Course:

A number of components of the projects have now been completed. Recruitment is taking place in the insulin, vasopressin, fluvoxamine parts as detailed in major findings section. A detailed analysis of the CSF variables with correlations being made with plasma and urine monoamine concentrations, will be made over the next year. These will be compared in relation to drinking history and type of alcoholism.

Publications:

Roy A, DeJong J, Adinoff B, Barbaccia M, Costa E, Guidotti A, Linnoila M. CSF diazepam-binding inhibitor in alcoholics and normal controls, Psychiatry Res 1990;31:261-6.

Adinoff B, Martin PR, Eckardt MJ, Bone GAH, Gold PW, Linnoila M. Pituitary-adrenal responses to oCRH and central neuropeptide levels in alcohol amnestic disorder, Biol Psychiatry 1991;29:1153-5.

Nutt DJ, Cowen PJ. Neurochemical studies on human volunteers, Pharmaceutical Med, in press.

Roy A, DeJong J, Adinoff B, Linnoila M. Acting-out in normal volunteers, Psychiatric Res, in press.

Roy A, DeJong J, Gold P, Rubinow D, Adinoff B, Ravitz B, Waxman R, Linnoila M. CSF somatostatin, CRH and ACTH in alcoholics and controls, Acta Psychiatr Scand, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 AA 00249-08 LCS

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Pharmacologic Reduction of Alcohol Consumption in Alcoholic Patients

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	D. George	Acting Section Chief	LCS, NIAAA
Others:	M. Eckardt	Acting Chief	LCS, NIAAA
	R. Eskay	Research Physiologist	ADAMHA
	M. Linnoila	Scientific Director	NIAAA
	N. Salem, Jr.	Section Chief	LCS, NIAAA

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Clinical Studies

SECTION
Section of Clinical Science

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NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL MAN-YEARS: .02	PROFESSIONAL: .02	OTHER:

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Recent studies indicate that alcohol consumption is regulated by several interacting neurotransmitters, including the dopamine and serotonin systems. In a randomized double-blind design, chronic alcoholic outpatients received L-DOPA or L-5-hydroxytrytophan, both with the peripheral decarboxylase inhibitor carbidopa or placebo for a one year period. During this year, alcohol consumption, liver function, craving for alcohol, mental status, psychosocial functioning, and compliance with medication were assessed at regular intervals. Prior to entry into the study, after 3 months, and at one year, the following procedures were conducted to measure drug effects: (1) behavioral evaluation; (2) determination of concentrations of drugs, monomanias, hormones, and peptides in blood and cerebrospinal fluid; (3) orthostatic changes in heart rate, blood pressure, and plasma norepinephrine concentrations; and (4) assessment of plasma vasopressin response to saline infusion. Changes in alcohol consumption will be related to biochemical and behavioral parameters.

This study has been completed. The results are being analyzed and have been submitted for publication.

Project Description:

Investigators:

D. George	Acting Section Chief	LCS, NIAAA
M. Eckardt	Acting Chief	LCS, NIAAA
R. Eskay	Research Physiologist	LCS, NIAAA
M. Linnoila	Scientific Director	NIAAA
N. Salem, Jr.	Section Chief	LCS, NIAAA

Objectives:

Over 90% of physicians in private practice prescribe drugs for the treatment of alcoholism, although valid studies demonstrating the efficacy of pharmacotherapy in this disorder are lacking. Successful treatment outcome may be defined as one or more of the following (1) reduction of the amount of alcohol consumed, (2) retention of the patient in treatment; (3) improvement of social and family relations; (4) maintenance of employment and financial status; and (5) amelioration of the medical and psychiatric complications of excessive alcohol consumption. There is now considerable evidence that reduction in alcohol consumption is of fundamental importance and can be expected to decrease the frequency and severity of alcohol-induced organic disease and to favorably modify behavioral problems that lead to or result from excessive alcohol consumption. Since alcohol consumption is maintained by reinforcement, which has a neurochemical basis, it may be possible to modify drinking behavior by pharmacologic interventions that alter central neurotransmitter function. Recent studies indicate that both central dopaminergic and serotonergic mechanisms may influence ethanol consumption. Evidence for functional dopaminergic and/or serotonergic deficits in alcoholics suggest that therapeutic strategies using the dopamine precursor L-DOPA and/or the serotonin precursor 5-hydroxytryptophan may benefit some patients.

Method Employed:

Alcoholic outpatients received L-DOPA or 5-hydroxytryptophan (both with the peripheral decarboxylase inhibitor carbodopa) or placebo, for a one year period in a randomized double-blind design. Alcohol consumption was monitored by a drinking logbook compiled by the patient, interview questionnaires with the patient and a significant other, sequential serum gamma GT levels, and breathalyzer readings at the time of clinic visits.

A lumbar puncture for measurement of biogenic amines and peptides, orthostatic norepinephrine test, and plasma vasopressin response to saline infusion prior to and after 3 months' treatment and one year's treatment allows neurochemical classification of patients and determination of whether patient and neurochemical characteristics are related to treatment outcome.

Major Findings:

Only eight (22%) of 37 patients were able to remain sober for one year. Most relapsed to a significant level of drinking within one or two months of entering the study. The administration of agents that augment dopamine (DA) and serotonin (5-HT) synthesis had minimal effects on the length of time patients remained sober. Alcoholic patients who remained sober for one year (irrespective of which treatment group they were assigned) had significantly lower concentrations of the DA metabolite, HVA at the start of the study compared to those who resumed drinking alcohol. Abstainers had a higher IQ during baseline evaluation, more education, and were less likely to show cognitive impairment.

Significance to Biomedical Research and the Program of the Institute:

The search for medications that can serve as adjuncts to counseling therapy in the treatment of alcoholism is a growing area of interest and importance.

Proposed Course:

The results from the study have been submitted for publication. Additional studies are planned studying the effects of dopamine on treatment outcome.

Publications:

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AA 00265-06 LCS

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Effects of Alprazolam, Diazepam, Clonidine, and Placebo Upon Ethanol Withdrawal

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	D. George	Acting Section Chief	LCS, NIAAA
Others:	M. Linnoila	Scientific Director	NIAAA
	K. Immerman	Clinical Associate	LCS, NIAAA

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Clinical Studies

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	2.5	0.5

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The ethanol withdrawal syndrome, which is characterized by an increased activity of the noradrenergic system, is at present most commonly treated with diazepam or chlordiazepoxide, both conventional benzodiazepines. Alprazolam, a triazolobenzodiazepine, has been demonstrated to be efficacious in the pharmacotherapy of depression and anxiety disorders, in contrast to the conventional benzodiazepines. Alprazolam may have a particularly potent inhibitory action on the noradrenergic system. It can, therefore, be postulated that alprazolam may be an effective and specific treatment for the ethanol withdrawal syndrome. Clonidine, a conventional antihypertensive, has been used to successfully treat withdrawal from the opiates, and most recently, nicotine and alcohol. This study compares the effects of alprazolam, clonidine, diazepam, and placebo on: 1) the signs and symptoms of the ethanol withdrawal syndrome, and 2) the noradrenergic overactivity of the ethanol withdrawal syndrome.

This project has been terminated.

PHS 6040 (Rev. 1/84) GPO 914-918

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00266-06 LCS

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Relationship of Psychobiology to Psychopathology in Alcoholics

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	D. George	Acting Section Chief	LCS, NIAAA
Others:	M. Eckardt	Acting Chief	LCS, NIAAA
	D. Goldman	Section Chief	LCS, NIAAA
	M. Linnoila	Scientific Director	NIAAA

COOPERATING UNITS (if any)
Experimental Therapeutics Branch, NIMH (W. Potter)

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Laboratory of Clinical Studies

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Section of Clinical Science

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NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL MAN-YEARS: 3	PROFESSIONAL: 2	OTHER: 1

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Alcoholism and affective disorders frequently occur in the same individuals and in members of the same family. This association may represent the co-existence of two common disease entities due to chance or due to (a) alcoholism resulting from self medication of an underlying affective disorder, or (b) depression resulting from toxic effects of alcohol abuse. Studies have shown that alcohol may acutely improve the sense of affective well-being, but with continued intoxication this improvement may be reversed. Also, during chronic experimental intoxication, alcoholics not only become increasingly depressed but also more anxious.

In this protocol we propose to characterize certain biochemical aspects of depression and anxiety as they occur in alcoholic patients. To do this, we will examine cerebrospinal fluid and plasma for norepinephrine (lying and standing), urine for catecholamine metabolites and employ pharmacological challenge paradigms using lactate, isoproterenol and chlorimipramine.

Project Description:

Investigators:

D. George	Acting Section Chief	LCS, NIAAA
M. Eckardt	Acting Chief	LCS, NIAAA
D. Goldman	Section Chief	LCS, NIAAA
M. Linnoila	Scientific Director	NIAAA
W. Potter	Section Chief	CPB, NIMH

Objectives:

To elucidate relationships of alcoholism to other psychopathology by studying the phenomenology and neurochemical characteristics of depression and anxiety in alcoholics and their non-alcoholic family members.

Methods Employed:

Patients admitted to the study will meet DSM IIIR criteria of the American Psychiatric Association for alcohol dependence and will have been abstinent from alcohol for at least two weeks. An examination by a psychiatrist and the Schedule for Affective Disorders and Schizophrenia (SADS) administered by a research social worker, will be used to make the diagnosis of past or present major affective disorder or anxiety disorder in alcoholic patients according to DSM IIIR criteria. Age- and sex-matched control populations will consist of (1) normal volunteers, (2) abstinent alcoholic patients without a diagnosis of major affective disorder or anxiety, and (3) adult offspring of alcoholics between the ages of 18 and 75 without a diagnosis of alcoholism.

Cerebrospinal fluid will be examined for monoamines, their metabolites, and peptides. Pharmacological challenge paradigms employing isoproterenol and chlorimipramine will be used to challenge the adrenergic and serotonergic systems which have been implicated in depression and anxiety disorders. Lactate infusions, commonly employed to induce panic in susceptible individuals, will be used as a probe for anxiety. Chloride ion and placebo will be administered in a double blind design concurrently with lactate to observe the effects of chloride ion on anxiety.

Major Findings:

To date, 72 patients have completed the lactate infusion study (21 alcoholics with panic disorder, 22 patients with panic). Preliminary results showed alcoholics with panic are less likely to panic during a lactate infusion compared with children of alcoholics with panic. The panic response obtained in panic patients is consistent with that reported for panic patients in the literature. Lactate induced physiological changes (BP, pulse) as well as biochemical changes (pH, electrolytes, lactate levels) showed no difference between the two groups thus yielding no obvious explanation for the reduced lactate response in alcoholics with panic. Of interest, both patients and controls show a decrease in vagal tone (a measure of parasympathetic activity) in response to the lactate infusion. Thirdly, it appears that panic subjects are less likely to have a lactate-induced panic attack if lactate is dissolved in D5W that if it is administered in NaCL. Isoproterenol and chlorimipramine infusions were administered to 20 alcoholics, 12 alcoholics with panic, 8 depressed alcoholics and 15 controls. The results of the isoproterenol infusion show there is no significant difference in either heart rate response or change in BP. Biochemical measures (norepinephrine cAMP and isoproterenol levels) await analysis. Chlorimipramine infusions were analyzed for changes in prolactin, cortisol and ACTH. Our results show no statistical difference between groups in hormonal response to chlorimipramine. Behaviorally subjects with panic disorder demonstrated a heightened affective response to chlorimipramine e.g. more anxiety and/or depression compared to alcoholics and controls.

Ten alcoholics, with and without panic, plus 8 ACOA's had lumbar punctures. Results show alcoholics with panic have statistically higher concentrations of B-endorphin (covaried for height, age, weight) compared to alcoholics without panic and controls. Lying/standing determinations of pulse, BP and norepinephrine levels show a trend toward reduced pulse response with standing and a concomitant increased production of norepinephrine in alcoholics with panic compared to alcoholics without panic.

The relationship between clinical variables and cerebrospinal-fluid (CSF) metabolites have recorded a number of interesting findings. Alcoholics with onset of heavy drinking before 20 years of age had significantly more antisocial personality traits, drug abuse, bipolar disorder, panic disorder, suicide attempts, and paternal alcoholism than alcoholics with onset after age 20 years. Alcoholics with onset before and after 20 years of age also differed significantly from each other for cerebrospinal fluid concentrations of diazepam-binding inhibitor and somatostatin. Depressed, compared with never-depressed alcoholics, had a higher daily alcohol intake, more lifetime diagnosis of other anxiety and affective disorders and drug abuse, more had attempted suicide, and more reported alcoholism in both parents. Depressed alcoholics also had significantly lower cerebrospinal fluid levels of dopamine metabolite homovanillic acid and of y-aminobutyric acid.

Significance to Biomedical Research and the Program of the Institute:

Alcohol abuse may have an adverse affect on mood contributing to feelings of anxiety, depression and suicidal ideation. Frequently, in order to cope with these negative emotional states, the alcoholic increases his drinking which results in a compromised physical and psychological state. To date, there have been a few studies which have attempted to study the biochemical links between anxiety and mood disorders and alcoholism. By understanding possible biochemical perturbations that arise from or contribute to alcoholism, we can gain a better understanding of the effects of alcohol and provide avenues for more effective pharmacological intervention.

Our results, which show that alcoholics with panic disorder have a reduced panic response to lactate, suggest the mechanism of panic disorder in alcoholics may have a different biochemical basis from that found in panic patients without alcoholism.

The drop in parasympathetic activity (measured as a decrease in vagal tone) in response to lactate suggests an imbalance in the autonomic nervous system may be having a role in the etiology of panic disorder. The lack of difference between controls and alcoholics with and without panic disorder in response to isoproterenol supports the conclusion that peripherial B- adrenergic receptors are not abnormal either in alcoholics or alcoholics with panic disorder. Similarly, the lack of neuroendocrine difference between groups in response to a chlorimipramine challenge suggests post-synaptic serotonin receptor function is not altered in alcoholics. The observation that subjects with panic disorder have a heightened anxiety response to chlorimipramine administration is interesting and warrants further investigation.

Relationships between clinical variables, and CSF metabolites suggest that age of onset may delineate subgroups of male alcoholics with significant clinical and biological differences.

Proposed Course:

Additional studies are being instituted to extend the knowledge gained from the lactate and chlorimipramine results. Procaine, a local anesthetic agent which has been shown to selectively stimulate the limbic structures of the brain, will be used to explore the possibility that repeated alcohol withdrawals may kindle certain individuals to heightened states of anxiety. Secondly, m-CPP, a selective serotonin receptor agonist, will be employed as an additional probe to study serotonin receptor function in alcoholics.

Publications:

Roy A, DeJong J, Lamparski D, Adinoff B, George T, Moore V, Kerich M, Linnoila M. Mental disorders among alcoholics: relationship to age of onset and cerebrospinal fluid neuropeptides, Arch Gen Psychiatry 1991; 48:423-27.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AA 00273-02 LCS

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Effects of Serotonergic Activity on Neuroendocrine & Behavioral Measures

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	D. George	Acting Section Chief	LCS, NIAAA
Others:	M. Linnoila	Scientific Director	NIAAA

COOPERATING UNITS (if any)
Laboratory of Clinical Studies, NIMH (D. Murphy)

LAB/BRANCH
Laboratory of Clinical Studies

SECTION
Section of Clinical Science

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NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL MAN-YEARS: 1.0	PROFESSIONAL: 0.5	OTHER: 0.5

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Several studies suggest possible serotonergic involvement in the neurobiology of alcoholism. To evaluate this possibility we administered the serotonin agonist m-chlorophenylpiperazine (m-CPP) to alcoholics, and controls. By observing the drug-induced behavioral effects and measuring changes in prolactin, cortisol and ACTH we hope to make inferences about post-synaptic serotonin function in subjects with alcoholism. Our results indicate alcoholics with an early onset of drinking-related problems (Type II) are statistically more likely to "desire a drink of alcohol" following IV administration of m-CPP than late onset alcoholics (Type I) or controls. This desire does not appear to be the result of drug induced anxiety.

Project Description:

Investigators:

D. George	Acting Section Chief	LCS, NIAAA
M. Linnoila	Scientific Director	NIAAA
D. Murphy	Chief	LCS, NIMH

Objectives:

There is a growing literature which suggests that brain serotonin (HT) may play a role in a variety of functions including mood, memory, appetite regulation, aggression and impulse control; areas of function frequently disturbed in patients with alcoholism. Pretreatment with the 5HT depleter PCPA or lesions of the raphe nuclei in the rat have, respectively, been reported to alter ethanol discrimination and delay the development of tolerance. Of special interest, a large series of selective 5HT agonists and antagonists administered to ethanol trained rats using a drug discrimination paradigm showed the 5HT 1B agonist TFMPP substituted for ethanol. This finding, in conjunction with recent clinical trials showing clinical efficiency of certain serotonin receptor agonists to reduce alcohol consumption, support the hypothesis that the disease process of alcoholism may be partially mediated through a central 5HT system. Since m-CPP has been widely used in other clinical studies at NIMH, and has been shown to possess a similar pharmacological profile to TFMPP, we administered m-CPP to alcoholics and alcoholics with panic disorder to investigate the resulting behavioral and pharmacologic effects.

Methods:

Prior to participation in the study, all subjects were carefully screened to assure they were in good physical health and conformed to predetermined diagnostic criteria. During the study subjects received an infusion of placebo followed by an intravenous dose of m-CPP. Individuals were carefully monitored for drug-induced changes in physical measures (pulse, blood pressure, vagal tone), mood and hormones (prolactin, ACTH, cortisol).

Major Findings:

Data has been collected on 15 Type I, 20 Type II and 22 control subjects. Major findings include: (1) Behaviorally Type II alcoholics were statistically more likely to "crave" alcohol following m-CPP compared with Type I alcoholic patients or controls. (2) Type I alcoholics experienced statistically more drug induced anxiety and dysphoria compared with Type II alcoholics and controls. (3) Biochemically m-CPP gave rise to a statistically greater rise in plasma ACTH in controls compared with Type I and Type II alcoholics.

Significance to Biomedical Research and the Program of the Institute:

Our results lend additional support to the possible involvement of serotonin in the pathophysiology of alcoholism. Presently we are in the process of designing new studies to better characterize the mechanism of how m-CPP can result in alcohol-like effects. It is hoped that through an understanding of this mechanism new treatment modalities can be introduced.

Publications:

Benkelfat C, George DT, Murphy DL, Hill JL, Nutt D, Linnoila M. Ethanol-like properties of the serotonergic partial agonist m-chlorophenylpiperazine in chronic alcoholic patients, Arch Gen Psychiatry 1991;48:383.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00274-03 LCS

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Intravenous Procaine in Alcoholics and Adult Children of Alcoholics

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	D. George	Acting Section Chief	LCS, NIAAA
Others:	M. Linnoila	Scientific Director	NIAAA

COOPERATING UNITS *(if any)*
Biological Psychiatry Branch, NIMH (R. Post); Clinical Brain Disorders Branch, NIMH (R. Coppola)

LAB/BRANCH
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SECTION
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TOTAL MAN-YEARS: 3.0	PROFESSIONAL: 2.5	OTHER: 0.5

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Previous studies show that the administration of procaine to bipolar and borderline patients gives rise to a diversity of behavioral responses ranging from euphoria to dysphoria. Since procaine is believed to selectively stimulate the limbic structures of the brain, it is reasoned that these behavioral differences might result from neuroclinical differences in these regions of the brain. Given that alcohol might alter limbic structures, we administered procaine to alcoholics, subjects with panic disorder, panic subjects with alcoholism and controls. Preliminary analyses of our results show alcoholics with panic disorder were more likely to experience a panic attack following procaine than were alcoholics without panic disorder or controls. Similarly, alcoholics with panic disorder had a greater rise in pulse per unit change in vagal tone compared to these other groups. Presently, there is insufficient data concerning panic subjects without alcoholism to draw any conclusion about this subgroup.

Project Description:

Investigators:

D. George	Acting Section Chief	LCS, NIAAA
R. Coppola	Senior Engineer	CBDB, NIMH
M. Linnoila	Scientific Director	NIAAA
R. Post	Chief	BPB, NIMH

Objectives:

The present study will address the hypothesis that abstinent alcoholics who have experienced a number of withdrawal bouts might have abnormalities of limbic function that would be revealed by procaine infusion. In addition, it is possible that temporal lobe dysfunction and kindling processes may occur in patients with panic disorder. In our experience a large number of alcoholics have had panic attacks. We shall, therefore, study a group of alcoholics who have panic disorder as well as alcoholism. To eliminate the possibility that any changes found might reflect predisposition to alcoholism, a sample of adult nonalcoholic offspring of alcoholics will also be recruited and tested. These will comprise a group that have had panic attacks and a group that is free of them.

Methods Employed:

Prior to participation in the study, all subjects will be carefully evaluated to assure they are in good physical health and conform to predetermined diagnostic criteria. During the study subjects receive placebo followed by two intravenous doses of procaine hydrochloride. Individuals are then carefully monitored for drug-induced changes in physical measures (pulse, blood pressure, vagal tone), mood, hormones (prolactin, ACTH, cortisol) and EEG activity.

Major Findings:

To date, 24 alcoholics, 14 controls and 11 alcoholics with panic disorder and 6 subjects with panic disorder but without alcoholism have been studied. Preliminary results show there is a statistically significant difference in the behaviorally induced effects of procaine between controls, alcoholics, and alcoholics with panic disorder. Alcoholics with panic disorder were more likely to experience a panic attack following procaine than were alcoholics without panic disorder or controls. Alcoholics with panic had a greater rise in pulse per unit change in vagal tone compared to either controls or alcoholics without panic disorder. Presently, there is insufficient data concerning panic subjects without alcoholism to draw any conclusions.

Significance to Biomedical Research and the Program of the Institute:

Our results suggest procaine may be a useful probe to characterize CNS differences between alcoholics and alcoholics with panic disorder. Similarly the differential effect in the relationship between pulse and vagal tone evidenced between groups, suggests that alcoholics with panic have altered parasympathetic autonomic control. The issue of kindling will be addressed when the EEG data are analyzed.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00278-02 LCS

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Behavioral Psychobiological Effects on 2-Deoxyglucose Infusions

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	D. George	Acting Section Chief	LCS, NIAAA
Others:	T. Alim	Senior Staff Fellow	LCS, NIAAA
	M. Linnoila	Scientific Director	NIAAA

COOPERATING UNITS *(if any)*
Clinical Neuroscience Branch, NIMH (D. Pickar)

LAB/BRANCH
Laboratory of Clinical Studies

SECTION
Section of Clinical Science

INSTITUTE AND LOCATION
NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3	2	1

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

2-deoxyglucose (2-DG) is a glucose analog which competitively inhibits glucose-6-phosphate dehydrogenase and leads to intracellular glucoprivation. In previous studies, 2-DG has been used as a stressor to activate both the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic adrenal axis and stimulate the appetitive centers of the hypothalamus. Our interest in this paradigm was generated by our clinical observation that alcoholics frequently consume increased amounts of carbohydrates following cessation of drinking. In order to explore possible hypothalamic abnormalities in subjects with alcoholism, we administered 2-DG to abstinent alcoholics that were classified into Type I and Type II subgroups using von Knorring's criteria. In this study we explored the behavioral and physiological changes arising from the 2-DG challenge. We postulated that a 2-DG induced glucoprivic response would give rise to both neuroendocrine and behavioral changes that might elucidate mechanisms of alcohol's action in the hypothalamus. Preliminary analysis of the results show alcoholics consume less calories than controls following both 2-DG and placebo administration. However, of interest alcoholics consume more carbohydrate than controls. The neuroendocrine responses are currently being assayed.

Project Description:

Investigators:

D. George	Acting Section Chief	LCS, NIAAA
T. Alim	Senior Staff Fellow	LCS, NIAAA
M. Linnoila	Scientific Director	LCS, NIAAA
D. Pickar	Chief	CNSB, NIMH

Objectives:

The study is designed to develop a stress paradigm that will allow us to study the effects of glucoprivation on behavior and neuroendocrine control. Clinical experience suggests that upon cessation of ethanol ingestion many alcoholics exhibit an increase in carbohydrate ingestion. In this regard since alcohol is also a carbohydrate it is possible that alcohol may be substituted for conventional carbohydrates. In this protocol we are interested if intracellular glucoprivation may serve as a internal "cue" that triggers and "urge to drink" alcohol.

Methods:

Prior to participation in the study, all subjects are carefully evaluated to assure they are in good physical health and conform to predetermined diagnostic criteria. During the study subjects receive placebo and two intravenous doses of 2-DG in random order. The subjects are carefully monitored for drug-induced changes in physical measures (pulse, blood pressure, vagal tone), mood, and hormones (prolactin, ACTH, cortisol).

Results:

Data has been collected on 26 alcoholics and 17 normal volunteers. Preliminary analysis performed on a subset of these patients show: (1) Alcoholics consumed less calories than controls following placebo and 2-DG. (2) Behaviorally, alcoholics reported greater sadness and confusion on the visual analogue. (3) Physiologically, both groups showed an increase in plasma glucose at the highest dose of 2-DG. (4) Biochemically changes in ACTH, prolactin and cortisol are currently being analyzed.

Significance to Biomedical Research and the Program of the Institute:

There is a growing literature reporting that carbohydrate consumption may play a role in mood regulation. In this protocol we explore the possibility that changes in intraneuronal glucose concentrations may trigger an urge to consume alcohol in patients with alcoholism.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 AA 00286-02 LCS

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Psychobiology of Alcoholism in Women

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	D. George	Acting Section Chief	LCS, NIAAA
Others:	T. Alim	Senior Staff Fellow	LCS, NIAAA
	M. Linnoila	Scientific Director	NIAAA

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Clinical Studies

SECTION
Section of Clinical Science

INSTITUTE AND LOCATION
NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL MAN-YEARS: .50	PROFESSIONAL: .50	OTHER:

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Studies suggest the pathophysiology of alcoholism may differ significantly between men and women. To explore this possibility we have employed several pharmacological challenge paradigms to compare the behavioral and endocrine responses between male and female alcoholics. Preliminary analysis of our results show that the m-CPP gives rise to minimal behavioral effects. This corresponds to the response obtained from Type I male alcoholics (late onset) demonstrated in a separate study. In contrast, none of the women experienced a m-CPP induced "desire to drink" response demonstrated to occur in approximately 40% of the Type II (late onset) male alcoholics.

Project Description:

Investigators:

D. George	Acting Section Chief	LCS, NIAAA
T. Alim	Senior Staff Fellow	LCS, NIAAA
M. Linnoila	Scientific Director	NIAAA

Objectives:

In the past, many of the conclusions derived from alcohol research have either been based on male populations or have not specifically analyzed the data according to gender. However, it is becoming increasingly clear that alcoholism in women may have a different clinical course compared to that in men. For example, population studies show women develop problem drinking later in life compared to men, while at the same time experiencing many of the complications of alcoholism, i.e. cirrhosis and malnutrition, at approximately the same age as men. These differences highlight the need to study alcoholism specifically in women.

Methods Employed:

Prior to participation in each study, all subjects were carefully screened to assure they are in good physical health and conform to predetermined diagnostic criteria. The following paradigm has been employed: m-chlorophenylpiperazine (m-CPP) challenge: m-CPP is a relatively selective serotonin agonist which we have been studying on our research ward. In males, m-CPP gives rise to a "high" and an "urge to drink" in approximately 50% of the Type II alcoholics, but rarely in Type I alcoholics. We are investigating effects of m-CPP in female alcoholics.

Major Findings:

To date, we have administered m-CPP to 7 women with alcoholism. Behaviorally, the women described mild anxiety symptoms which were similar to those described by Type I male alcoholics. No one described a "high" or "urge to drink" which had been described by approximately 40% of Type II male alcoholics.

Significance to Biochemical Research and the Program of the institute:

By studying parameters related to serotonin function, carbohydrate metabolism and hypothalamic control, we hope to gain new insights into the pathogenesis of alcoholism in women.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00233-09 LCS

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Family Studies of Alcoholism

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	G. Brown	Unit Chief	LCS, NIAAA
Others:	I. Culver	Psychologist	LCS, NIAAA
	L. Doty	Social Worker	LCS, NIAAA
	S. Goodson	Senior Staff Fellow	LCS, NIAAA
	C. Jones	Psychology Technician	LCS, NIAAA
	V. Moore	Social Worker	LCS, NIAAA
	M. Trunzo	Social Worker	LCS, NIAAA

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Clinical Studies

SECTION
Section of Clinical Science, Unit of Family Studies

INSTITUTE AND LOCATION
NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL MAN-YEARS: 4.0	PROFESSIONAL: 4.0	OTHER: 0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The Unit of Family Studies has two major functions (1) to recruit and assess alcoholics, controls and their families, for various investigators within the Laboratory of Clinical Studies; and (2) to conduct psychosocial and psychobiological studies of alcoholic families and their individual members. The Unit staff continues to focus on coding and entering onto a computer the data collected since the inception of the Laboratory. A series of correlational studies comparing suicidal versus non-suicidal alcoholics on clinical, psychosocial and family variables has been carried out. In addition, the Unit has collected data on the adult offspring of alcoholics and non-alcoholics, black and white, respectively; pilot efforts for a follow-up of matched black and white former patients of our inpatient unit has begun. Unit staff has also been collaborating with the Section on Genetic Studies in identifying and phenotyping several pedigrees for linkage analysis. Clinical expertise in the psychiatric assessment of children has been developed.

Project Description:

Investigators:

G. Brown	Unit Chief	LCS, NIAAA
I. Culver	Psychologist	LCS, NIAAA
L. Doty	Social Worker	LCS, NIAAA
S. Goodson	Senior Staff Fellow	LCS, NIAAA
C. Jones	Data Technician	LCS, NIAAA
V. Moore	Social Worker	LCS, NIAAA
M. Trunzo	Social Worker	LCS, NIAAA

Objectives:

To accumulate and evaluate clinical and genetic data through comparative studies of alcoholic and normal control patients and their respective family members including children. To investigate the role of genetic and environmental influences through: (1) studies of multigenerational families, family process, and family systems, and their relation to the maintenance of abusive and addictive drinking behavior; (2) phenomenological studies of subgroups of alcoholics (women and blacks); (3) comparisons between different alcoholic populations (i.e., those who attempt suicide vs. those who do not; those who become violent while drinking vs. those who do not); (4) longitudinal studies of children at risk, focusing on predictive factors and early detection; and (5) systematic studies of outcome.

Method Employed:

Assessment and data collection methodology includes use of the following: SADA-L, SCID, SCID II, DICA-C/A, DICA-PRDC-F, MMPI, Michigan Alcoholism Screening Test, and Races-III (Olsen), as well as other instruments which will enable us to quantify psychosocial and psychobiological characteristics. Expertise has been developed with software for computer-assisted interviews for some of the above clinical assessments.

Major Findings:

Previous work has included studies of Type I and Type II alcoholics, comorbidity of alcoholism and major depressive disorders and suicidal behavior, and correlations studies with cerebrospinal fluid metabolites. Current work in progress includes childhood psychiatric assessment and EEG variables and personality traits and Axis II diagnoses in adults. Early pilot attempts to match black and white former inpatients has demonstrated the difficulty of locating and securing the cooperation of patients who have not been seen for several years. Many investigators in the NIAAA intramural program who make use of clinical variables obtained directly by interview or self-administered clinical or behavioral instruments are collaborators with this Unit. Most of the publications in which we participate are reported by other laboratories and sections within the NIAAA intramural program. All data are now preliminary.

Significance to Biomedical Research and the Program of the Institute:

By using our extensive demographic and clinical data base, we hope to expand our ability to classify alcoholics into meaningful subgroups. This will hopefully allow us to develop hypotheses related to the etiology and pathophysiology of alcoholism as well as more specific and efficacious treatment approaches.

Publications:

Roy A, DeJong J, Lamparski D, George T, Linnoila M. Depression among alcoholics: relationship to clinical and cerebrospinal fluid variables, Arch Gen Psychiatry 1991;48:428-32.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AA 00279-02 LCS

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Black and White Offspring of Parental Alcoholics

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	V. Moore	Social Worker	LCS, NIAAA
Others:	G. Brown	Unit Chief	LCS, NIAAA
	I. Culver	Psychologist	LCS, NIAAA
	M. Linnoila	Scientific Director	NIAAA

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Clinical Studies

SECTION
Section of Clinical Science, Unit of Family Studies

INSTITUTE AND LOCATION
NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
.35	.30	.05

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This study compares Black and White adult offspring from middle class families with parental alcoholism (ACOA - adult children of alcoholics) and without parental alcoholism (ACONA - adult children of non-alcoholics) with age similar sex and race matched controls. Findings include a greater frequency of both alcoholism and major depression in ACOA when compared with ACONA. Males are at higher risk for alcoholism while females may be at higher risk for major depression . No racial differences are apparent.
Sex of alcoholic patient appears not to be predicative for offspring. Negative family history for both first degree and second degree relatives is most closely associated with no alcoholism or other psychiatric disorder in index offspring.

Project Description:

Investigators:

V. Moore	Social Worker	LCS, NIAAA
G. Brown	Unit Chief	LCS, NIAAA
I. Culver	Psychologist	LCS, NIAAA
M. Linnoila	Scientific Director	NIAAA

Objectives:

Though some studies of the offspring of alcoholics have been published, none have controlled or differentiated age, sex, and racial (black/white) variables in the same way as this study. Particularly of interest are differences in psychiatric diagnoses. Research on the risk for development of psychopathology in children of alcoholics has focused on three major areas: genetics, teratogenic factors during pregnancy, and environmental factors. One important question generated from past research is whether the risk for developing psychopathology is the same for adult offspring of parental alcoholics (AOPA) vs. parental non-alcoholics (AOPNA). Common familial transmission of alcoholism, depression, and antisocial personality and the separate transmission of these disorders in families have been demonstrated in several studies. This preliminary report compares psychiatric diagnoses of AOPA vs. AOPNA taking into consideration age, sex, and race with the coincidence of alcoholism with other psychiatric diagnoses.

Methods Employed:

Black and white subjects of approximately equal numbers were of similar age (both mean and distribution), same sex and equally distributed among AOPA and AOPNA. All groups were predominantly middle class. Either one or both parents had received a diagnosis of alcoholism at NIAAA. Psychiatric diagnosis for both parents and offspring were determined by doubly blind-rated, SADS-L interviews, corroboration by relatives and/or other external sources (i.e., medical records). Prior to formal clinical assessment, telephone screenings by social workers and psychologists were also completed. Exclusion criteria for proband, but not for offspring, were 1) less than 18 years old, 2) regular drug use; more often than once a week or and/or i.v. use, 3) major medical problems; 4) an acute episode of severe antisocial behavior. SADS-L interviews were done by social workers, clinical psychologists, student interns under supervision, or staff psychiatrists. All interviewers' techniques were supervised by an experienced clinical psychologist or psychiatrist who were also one of the blind raters. These assessments have occurred over six years (1984-1990).

Major Findings:

Alcoholism, but not drug abuse is more likely to occur in AOPA than in AOPNA with men (no race differences) accounting for disproportionate share of the alcoholism in both groups. Both black and white AOPNA men drink more than black and white AOPA women, raising the possibility that the sex differences may be more important than parental drinking history. AOPNA women drink the least of any group. No racial differences are apparent. Furthermore, major depression is more likely to occur in AOPA vs. AOPNA. Drug abuse and anti-social personality have high coincidences with alcoholics, particularly in alcoholic males under age 25 whether or not the subject is AOPA or AOPNA. Those AOPNA with second degree relatives who are alcoholic have a significantly greater incidence of alcoholism and other psychiatric disorders than those AOPNA with no second degree relatives who are alcoholics.

Significant to Biomedical Research and the Program of the Institute:

This preliminary report indicates that both parental drinking history and sex are risk-factors for the development of alcoholism. Among the subjects studied here, race does not appear to be a particular risk-factor. Furthermore, since all subjects within race and across-race were of similar social class, with similar distribution, these findings of risk factors for alcoholism and psychiatric diagnoses, appear not to be confounded by social and cultural factors. These data do not make any distinction between genetic and learning influences on AOPA or AOPNA. Coincidence of alcoholism with other psychiatric disorders appears to be in agreement with the literature except for the low coincidence of major psychoses and axis II behavioral disorders particularly anti-social personality. This low incidence may be explained by the exclusion of these disorders in the parents of the offspring studied.

Proposed Course of Project:

This project needs some additional patients and further analysis on the familial data. Several initial clinical reports have been presented at meetings. Further biological assessments are also anticipated.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 AA 00290-01 LCS

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Studies on Families with Serotonin Gene Defects

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	M. Filling-Katz	Senior Staff Fellow	LCS, NIAAA
Others:	L. Akhtar	Chemist	LCS, NIAAA
	G. Brown	Unit Chief	LCS, NIAAA
	M. Eckardt	Acting Chief	LCS, NIAAA
	D. Goldman	Section Chief	LCS, NIAAA
	M. Linnoila	Scientific Director	NIAAA
	D. Nielsen	Senior Staff Fellow	LCS, NIAAA

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Clinical Studies

SECTION
Section on Genetic Studies

INSTITUTE AND LOCATION
NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL MAN-YEARS: 10	PROFESSIONAL: 6	OTHER: 4

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
- ☒ (a1) Minors
- ☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Studies on individuals with severe genetic defects in serotonin function can shed light on the role of this neurotransmitter in behavior and on the role of milder functional variants in serotonin genes in predisposing individuals to psychopathologies and to alcoholism. We have targeted for these studies patients with a positive family history or significant dysmorphology with severe mental retardation, pervasive developmental disorder, infantile autism, obsessive compulsive disorder, disruptive behavioral disorder or other disrupted behaviors which are presumptively under serotonergic influence. We are identifying probands for family studies by measuring the serotonin metabolite 5HIAA in cerebrospinal fluid. Probands and family members receive a detailed neurologic and psychiatric assessment and familial transmission and comorbidity is concurrently assessed. Subjects with aberrant serotonin function and their families will be the focus for genetic linkage analyses using dispersed probes and direct studies (for example sequencing) of candidate genes such as tryptophan hydroxylase.

Project Description:

Investigators:

M. Filling-Katz	Senior Staff Fellow	LCS, NIAAA
L. Akhtar	Chemist	LCS, NIAAA
G. Brown	Unit Chief	LCS, NIAAA
M. Eckardt	Acting Chief	LCS, NIAAA
D. Goldman	Section Chief	LCS, NIAAA
M. Linnoila	Scientific Director	NIAAA
D. Nielsen	Senior Staff Fellow	LCS, NIAAA

Objectives:

The role serotonin plays in developing and controlling behavior has not been completely defined, although the importance of this neurotransmitter in alcoholism and other psychopathologies is clear. In this study we are attempting to gain insights into the normal serotonin gene function and the effects of milder gene defects by identifying and studying individuals with severe defects in serotonin genes. Although serotonin modulates a number of important behaviors including sleep, impulsivity, appetite, and body temperature as well as ethanol intake, no human or animal behavioral mutants in serotonin function have been identified. It is therefore logical that abnormalities of serotonin function may cause unknown effects in adult tissues and during development. In this study we will 1) identify patients with biochemical genetic deficits in serotonin function, 2) assess the neurochemical, behavioral, neurophysiological, and medical manifestations of genetic serotonin dysfunction in patients and family members, 3) characterize genetic transmission of these traits in the families and 4) obtain DNA to serve as the source for the isolation of molecular defects in serotonin genes.

Methods employed:

Selection of probands

Children and adults with evidence of a serotonergic genetic disorder either by family history or the presence of significant dysmorphology are screened. Candidate serotonergic genetic disorders being screened include 1) idiopathic metal retardation (IQ<50), 2) pervasive developmental disorder, 3) infantile autism, 4) obsessive-compulsive disorder, 5) disruptive behavioral disorder and 6) other serotonin-associated behavioral disorders (impulsivity or severe eating disorders). A compete medical, neuropsychiatric and genetic evaluation is performed on these subjects. Electrophysiological assessment of baseline EEG and event related potentials (ERPs) are performed according to the patient's functional level. Monoamine and monoamine metabolite levels are obtained from cerebrospinal fluid. Magnetic resonance imaging of the brain and cognitive testing are performed if clinically indicated.

Psychiatric assessment is being performed in collaboration with Gerald L. Brown (Unit on Family Studies, LCS). This assessment process includes the Structured Clinical Interview for DSMIII-R (SCID) for adults, the Diagnostic Interview for Child Assessment (DICA) for children greater than 5 yrs of age and the Denver Developmental Assessment for younger children. Children less than 5 yrs of age or not suitable for the DICA receive an age-appropriate interview conducted by a psychiatrist. Tests of personality, mood and affect include the Child Behavior checklist, the Spielberger State Anxiety Scale, the Child Depression Inventory (for age <16 yrs), the Beck Depression Inventory (for age >16 yrs) and the Brown-Goodwin Scale (Brown, 1979). If appropriate, cognitive assessment is performed in collaboration with Michael Eckardt (LCS, NIAAA). Age appropriate tests for intelligence, cognition and memory include Wechsler Adult Intelligence Scale (WAIS-R), the Wechsler Intelligence Scale for Children (WISC-R), the Bailey Infant Scale and the Wide Range Achievement Test (WRAT).

Memory and cognition are assessed with the Matching Familiar Figures Test and Porteus Mazes. Motor tests include the Draw-a-line-slowly and Draw-a-line-fast tasks and various reaction time tasks. Electrophysiological evaluation will be performed in certain subjects and families, in part chosen according to subject's functional level. These studies will be in collaboration with Michael Eckardt (LCS, NIAAA) and will include event-related potentials. In the ERP paradigm, subjects are given trains of stimuli interspersed with occasional oddball stimuli. One of the elicited responses, the O wave, is thought to be influenced by serotonergic neurotransmission.

CSF measures of monoamines, monoamine metabolites, and neuropeptides are obtained after a standard 3 day low monoamine diet and lumbar puncture under carefully controlled conditions.

For DNA and protein genetic marker studies, venipuncture is performed and a lymphoblastoid cell line is established. Genetic linkage studies will be performed with dispersed markers chosen for informativeness and on candidate genes involved in serotonin function, such as tryptophan hydroxylase and serotonin receptor genes.

Major Findings:

Although behavior is ultimately controlled by numerous genes and other causative factors, better clinical subtyping and the study of highly abnormal individuals can result in the identification of discrete genetic entities. Our approach to find genetic phenotypes due to serotonin dysfunction has been to study individuals with abnormal behavioral traits or neurochemical abnormalities related to serotonin function. In this recently initiated research protocol, we have thus far studied two families with hereditary abnormalities which may be linked to serotonin function. The first family studied included two obligate carriers for Wolfram's syndrome, a syndrome marked by juvenile optic atrophy, diabetes mellitus, and diabetes insipidus, and by the behavioral problems of depression, psychosis and suicide. The second family has a high prevalence of psychosis (6 female family members) as well as alcoholism, gambling and criminality (4 male family members). Two of the females in this family have a syndrome including juvenile macular degeneration, pseudoxanthoma elasticum, demyelinating neuropathy, and generalized neurodegenerative disease of unknown origin.

Significance to Biomedical Research and the Program of the Institute:

Diminished turnover of serotonin is associated with impulsive behavior and appears to be related to alcoholism risk. Because of the diverse functions of serotonin in regulating sleep, appetite, mood and body temperature, it is highly likely that genetic variants of serotonin genes may be important in a variety of disorders such as obesity, affective illness and alcohol and drug abuse. We have chosen to study highly abnormal individuals and their families because the molecular pathology of extreme genetic phenotypes is more likely to be clear, leading to a better understanding of the relationship between serotonin function and behavior and increasing the likelihood that we can map the relevant genes.

Proposed Course:

We will analyze genes involved in serotonin function (tryptophan hydroxylase and the 5HT1A receptor) using the DNA collected from the two families studied thus far. We will extend these families and eventually will identify 50 probands and study 200 family members. Three additional families already identified for study include 1) a family with two male siblings with Pervasive Developmental Disorder and 2) a family with four manic depressives and a boy with mental retardation and severe conduct disorder. Using a comprehensive approach including neurobehavioral, anatomical and physiological analyses, we will characterize the phenomenology of these familial neurogenetic disorders and seek a relationship between the clinical features and the CSF neurochemical findings. In appropriate families, we will perform genetic transmission and linkage analyses.

Publications:

Goldman D, Filling-Katz M. Identifying genes determining trait differences in behavior and drug response, J Psychopharm 1991;5:11-7.

Filling-Katz MR, Choyke PL: Genetic mechanisms in von Hippel-Lindau disease, Lancet 1991;337:1477-8.

Seizinger B, Smith D, Filling-Katz M, et al. Genetic flanking markers refine diagnostic criteria and provide new insights into the genetics of von Hippel Lindau disease, Proc Natl Acad Sci (USA) 1991;88:2864-8.

Fink J, Ravin-McCrae P, Filling-Katz M, et al. Clinical and genetic aspects of diurnally variant dystonia of the Sagawa type, Arch Neurology, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00280-02 LCS

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Genetic Studies of the Electroencephalogram and Event-Related Potentials

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	D. Goldman	Section Chief	LCS, NIAAA
Others:	A. Bolos	Visiting Fellow	LCS, NIAAA
	G. Brown	Unit Chief	LCS, NIAAA
	M. Eckardt	Section Chief	LCS, NIAAA
	M. Filling-Katz	Senior Staff Fellow	LCS, NIAAA
	C. Fraser	Section Chief	LPPS, NIAAA
	E. Kirkness	Visiting Associate	LPPS, NIAAA
	V. Moore	Social Worker	LCS, NIAAA

COOPERATING UNITS *(if any)*
Laboratory of Viral Carcinogenesis, NCI (S. O'Brien); Program Resources, Inc., Frederick, MD (M. Dean); Washington University (J. Rohrbaugh); M. Enoch (London, England)

LAB/BRANCH
Laboratory of Clinical Studies

SECTION
Section on Genetic Studies

INSTITUTE AND LOCATION
NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
10	6	4

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The goal is to identify the genes determining genetic variants of the electroencephalogram (EEG) and of evoked responses of the EEG and to explore the relationship of these genes to behavior including psychopathologies such as alcoholism, drug abuse, antisocial personality, anxiety disorders and affective illness. This initiative is being pursued by: 1) family transmission and association studies on families identified by a population-based survey for probands with EEG variants 2) a genetic linkage study to map the genes determining the variants. In the genetic epidemiological study, the low voltage (LV) alpha, EEG variant was confirmed to be transmitted in autosomal dominant fashion. However, the monomorphic alpha resting EEG pattern was found to be largely sporadic in its distribution. Alcoholism and anxiety were increased in individuals with the LV trait. Families with the LV variant have been collected and expanded and are being analyzed for genetic linkage to dispersed and candidate gene DNA and protein markers.

Project Description:

Investigators:

D. Goldman	Section Chief	LCS, NIAAA
A. Bolos	Visiting Fellow	LCS, NIAAA
G. Brown	Unit Chief	LCS, NIAAA
M. Dean	Staff Scientist	PRI, Frederick, MD
M. Eckardt	Section Chief	LCS, NIAAA
M. Enoch		London, England
M. Filling-Katz	Senior Staff Fellow	LCS, NIAAA
C. Fraser	Section Chief	NIAAA
E. Kirkness	Visiting Associate	NIAAA
V. Moore	Social Worker	LCS, NIAAA
S. O'Brien	Chief	LVC, NCI
J. Rohrbaugh	Associate Professor	Washington Univ., St. Louis, MO

Objectives:

The main objective is to locate using "reverse genetics" genes responsible for alcoholism and associated behaviors. Genetic risk is an important determinant of alcoholism and other psychopathologies. Application of large numbers of available candidate and dispersed DNA probes as markers for genetic linkage analyses would seem to offer the clear possibility for identifying risk genes for these disorders. However to identify genes for complex, heterogeneous psychiatric diseases, it would be highly advantageous to define genetic psychiatric phenotypes with greater precision and to find phenotypes which correlate more precisely with genotype. One approach to refining the genetic behavioral phenotype is to type neurophysiologic variants relevant to behavior. In alcoholism and other psychopathologies, there is a diminished amplitude of the P300 evoked potential. Variants of the EEG, such as the low voltage alpha variant, have been shown to be transmitted in Mendelian fashion. Evidence indicates that alcoholics have an increased frequency of the low voltage alpha variant. We therefore wish to define the prevalence, interrelationships, significance to behavior including alcoholism, genetic transmission, and genetic linkage of variants of the EEG and variants of event-related potentials (ERPs).

Methods Employed:

Psychiatric assessment

Familial alcoholics and their families were clinically assessed in collaboration with Dr. Gerald L. Brown (Unit on Family Studies, LCS, NIAAA). Family members received a comprehensive clinical psychiatric and psychological evaluation, including the SADS-L psychiatric interview, life history of impulsive behavior, the MAST for alcohol and drug use, a structured family history and several psychological tests including the MMPI and EPQ (Eysenck Personality Questionnaire). Consensus clinical diagnoses were made by two raters blind to subject identities and incorporating data from corroborative family interviews and from other records.

Ascertainment of EEG families

For EEG genetic studies, we identified probands with genetic variants of the EEG and studied their families in detail. Subjects were screened for medical conditions or drug use which would interfere with analyses and derived from families with two parents and at least two adult siblings available for analysis. Probands were identified using methods for objective power spectrum and time series signal analyses developed by Drs. John Rohrbaugh, Michael Eckardt and others within the Section on Clinical Brain Research.

EEG and ERP measures

The EEG genetic analyses were performed in collaboration with Drs. Rohrbaugh and Eckardt of that Section. The EEG and ERP measures were derived from multiple sites with separate channels for the electrooculogram and heart rate.The resting EEG was performed for 10 minutes with eyes open, eyes closed and with the subject hyperventilating. The EEG was subjected to power spectral analysis and was also classified using the criteria of Vogel. In the ERP paradigm, subjects experienced two simultaneous trains of stimuli: auditory stimuli consisting of simple tones of varying pitch and visual stimuli derived from the 'heads' task developed by Begleiter et al. ERP's were recorded in two situations: in one, the subject identified occasional target visual stimuli whilst ignoring the simultaneous auditory stimuli, conversely in the second, the rare auditory target was the focus of attention. The paradigm therefore yielded P300s for both auditory and visual stimuli as well as a variety of additional ERP measures related to attention and cognition.

For DNA and protein genetic markers

A blood sample was obtained and a lymphoblastoid cell line established. Cells were cultivated to provide sufficient DNA and protein for marker studies and to cryopreserve cells.

For genetic linkage, we are testing for nonrandom assortment of marker and phenotype in families, linkage being detected if the two are transmitted as if coupled together or as if in repulsion. Because the likelihood of establishing linkage depends in part on the number of genetic markers typed, a large number of protein and DNA polymorphisms are used. On two-dimensional electrophoresis (2DE) protein gels of serum and of lymphoblasts, 25 markers are typed and the chromosomal location of the majority of these markers is now known. These polymorphisms, for the most part originally described by us, are variants in isoelectric point. The DNA polymorphisms include variants in the sites at which restriction endonucleases cut DNA (RFLPs) and variation in the length of cut fragments due to a variable number of copies of a single sequence repeated many times in a tandem array (VNTRs). In collaboration with Stephen O'Brien (NCI, Laboratory of Viral Carcinogenesis) and Michael Dean (Program Resources Inc, Frederick, MD) we are currently typing 200 DNA polymorphisms and have a much larger collection of probes which recognize polymorphisms.

Major Findings:

Genetic Linkage in Families with EEG and ERP variants

This study was designed to determine genetic transmission of hereditary EEG and event-related potential (ERP) traits which have been related to alcoholism, to further define their behavioral significances and ultimately to map genes determining these trait differences. Three phenotypes which we studied were the low voltage (LV) alpha domain of the resting EEG in which alpha (8-13 Hz) activity is absent or greatly diminished, the monomorphic alpha (M) resting EEG trait and the diminished amplitude P300 component of the ERP. Vogel had presented evidence that the LV and M traits had prevalences of approximately 4% and were transmitted in autosomal dominant fashion. Vogel proposed that individuals with the LV phenotype are more likely to become alcoholics. Vogel et al have also reported psychometric differences between LV and M individuals. However, these results as well as the population and genetic transmission results were unconfirmed. ERPs are at least in part genetically determined. Begleiter et al and others have found that adult alcoholics and children at risk for alcoholism have a diminished amplitude of the P300 component occurring after stimuli that engage certain types of cognitive activity. The diminished P300 may reflect an impairment in cognitive processing. Little was known about the relationship between P300 and personality traits or to resting EEG traits.

In collaboration with J. Rohrbaugh and M. Eckardt (Clinical Brain Research), Mary-anne Enoch determined that the population frequency of the M variant was 9% and the LV variant was present in 12.5%. The population sample, recruited through general advertising, had been screened for medical contraindications and drug use and consisted of adults with two living biological parents and at least three fully related adult children. Therefore it was possible to collect data for mode of transmission and for genetic linkage.

This study differed from previous ones in that it combined population genetic analysis with current methods of EEG spectral analysis to objectify and more accurately define EEG traits. Our results so far indicate that the LV variant is a discrete trait occurring in 12.5% of the population. The M variant was found in 9%. As postulated, the LV trait was increased in frequency in alcoholics and individuals with anxiety disorders. Neither trait was age-dependent.

Transmission analysis on a set of eight families indicated that the LV trait was transmitted as an autosomal dominant trait. The relative likelihood for autosomal dominant transmission versus no genetic transmission was >1017 assuming a genetic penetrance of 0.9 and a population prevalence of the trait of 4%.

When 7 families with monomorphic alpha probands were studied, a >2.7 x 102 relative likelihood for autosomal dominant transmission versus no transmission was generated for the family of the most extreme proband. However, the six other families provided evidence against genetic transmission, possibly indicating a role for genetic transmission in only the more extreme monomorphic alpha phenotype.

Molecular probes for genetic linkage

The goal of identifying genes whose variants underlie vulnerability towards alcoholism is being pursued in part through hypothesis driven, candidate locus studies (forward genetic approaches) but also by genetic linkage studies in families and well-defined populations (reverse genetic approaches). There is considerable interplay between the two approaches, for example genetic variation at candidate loci involving serotonin and GABA-benzodiazepine function is being sought in families also suitable for genetic linkage using random probes.

For candidate gene analyses, we have identified polymorphisms in the human 5HT1A receptor and, collaboratively with Drs. Clare Fraser and Ewen Kirkness (NIAAA), in the GABAB receptor gene. The GABAB polymorphism is detected by polymerase chain reaction of a region of the gene containing a tetranucleotide repeat. This GABAB receptor polymorphism was used to map that gene to the centromeric region of chromosome 4. As described in other project annual reports, we have also discovered DNA polymorphisms for human tryptophan hydroxylase, ADH5, and transketolase.

In the reverse genetic approaches, probes were chosen for informativeness rather than for function. Due to the collaborative effort with S. O'Brien (Laboratory of Viral Carcinogenesis, NCI) and M. Dean (Program Resources Inc., Frederick, MD), we are typing 200 restriction fragment length polymorphisms in families. By two-dimensional electrophoresis (2DE) of serum and lymphoblast proteins, 25 expressed markers are typed. These polymorphisms, for the most part originally described by us, are variants in isoelectric point. We have genetically mapped eight of the polymorphic 2DE lymphocyte proteins by typing them in a panel of nine CEPH families and establishing genetic linkage to mapped DNA polymorphisms. The majority of the serum and lymphocyte 2DE polymorphisms are now genetically mapped.

Significance to Biomedical Research and the Program of the Institute:

Genetically transmitted variants of the EEG are not uncommon. For example, the low voltage alpha variant is estimated to be present in 4% of the population and we found a higher frequency of the trait in subjects with alcoholism or with anxiety disorders. The genetically complex psychopathologies which can be addressed using these EEG markers are also relatively common and have a profound impact on population mortality, productivity and quality of life.

Linkage studies in humans

Familial linkage studies offer a direct method of identifying genetic determinants and markers for vulnerability to alcoholism, bridging the gap between behavioral phenotypes and molecular mechanisms. They also offer a definitive method for testing specific hypotheses regarding the effects of variants at candidate genetic loci including neurotransmitter biosynthetic, receptor and alcohol metabolic loci.

EEG

The clinical and electrophysiological phenotyping of families has allowed us to identify an EEG phenotype which is transmitted in autosomal dominant Mendelian fashion and which shows some relationship to alcoholism, anxiety and depression. The cell lines we have prepared from the members of these families offer a permanent source of DNA for the linkage analyses which can help us to identify a gene for this trait.

Proposed Course:

We will continue to type candidate and random dispersed genetic markers in families with the low voltage alpha variant of the EEG in order to identify a gene or genes determining this trait.

Collection of families with EEG variants which have been related to alcoholism will continue to provide additional material for linkage analyses and for studies on genetic transmission and quantitative gene effects on EEG parameters.

We will further assess the interrelationship between different variants of the EEG and ERP and the relationships of these variants to behavioral traits including alcoholism.

Publications:

Limson R, Goldman D, Roy A, Lamparski D, Ravitz B, Adinoff B, Linnoila M. Personality and CSF monoamine metabolites in alcoholics and controls, Arch Gen Psychiatry 1991;48:437-41.

Janczewski DN, Goldman, D, O'Brien, SJ. Molecular genetic distance estimates between orangutan (Pongo pygmaeus) subspecies based on isozyme and two-dimensional electrophoresis, J Heredity 1990; 81:375-87.

Goldman D, O'Brien SJ, Lucas S, Dean M. Genetic linkage of eight polymorphic candidate loci for immune variation, Genomics, in press.

Dean M, Lucas-Derse S, Bolos A, O'Brien SJ, Kirkness EF, Fraser CM, Goldman D. A highly informative 4bp microsatellite locus in the GABARb1 receptor gene and its localization on chromosome 4p, Am J Hum Genet, in press.

O'Brien SJ, Benveniste RE, Nash WG, Martenson JS, Eichelberger MA, Wildt DE, Bush M, Wayne RK, Goldman D. Molecular biology and evolutionary theory: the giant panda's closest relatives. In: Liss AR, Warren L, Meselson M, eds. New Perspectives on Evolution. New York:1990;225-50.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00281-01 LCS

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Molecular Genetic Studies on Alcoholism in American Indians

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	D. Goldman	Section Chief	LCS, NIAAA
Others:	A. Bolos	Visiting Fellow	LCS, NIAAA
	G. Brown	Unit Chief	LCS, NIAAA
	M. Linnoila	Scientific Director	NIAAA
	R. Robin	Senior Staff Fellow	LCS, NIAAA

COOPERATING UNITS *(if any)*
Laboratory of Viral Carcinogenesis, NCI (S. O'Brien); Program Resources Inc., Frederick, MD (M. Dean); Indian Health Service, Clinton OK (B. Albaugh), Univ. of New Mexico (J. Long, F. Romero), PECR, NIDDK (P.Bennett, W. Knowler).

LAB/BRANCH
Laboratory of Clinical Studies

SECTION
Section on Genetic Studies

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TOTAL MAN-YEARS: 10
PROFESSIONAL: 6
OTHER: 4

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☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

To identify alcoholism risk genes, we are collecting and testing for linkage families from three American Indian populations which are relatively homogeneous and in which alcoholism is highly prevalent. This study also addresses the genetic epidemiology and psychiatric comorbidity of alcoholism in Indians. Two hundred Cheyenne Indians have been clinically evaluated and their cell lines immortalized. At Jemez Pueblo, data and cell lines have been collected for 100 subjects. A study on the Pima Indians is in progress. A large panel of random polymorphic probes has been typed in Cheyenne alcoholics and controls and genetic linkage analysis is in progress for this group.

Project Description:

Investigators:

D. Goldman	Section Chief	LCS, NIAAA
B. Albaugh	Dir. Soc. Services	IHS, Clinton, OK
P. Bennett	Chief	PECR, NIDDK
A. Bolos	Visiting Fellow	LCS, NIAAA
G. Brown	Unit Chief	LCS, NIAAA
M. Dean	Staff Scientist	PRI, Frederick, MD
W. Knowler	Section Chief	PECR, NIDDK
M. Linnoila	Scientific Director	NIAAA
J. Long	Assoc. Prof.	UNM
S. O'Brien	Chief	LVC, NCI
R. Robin	Senior Staff Fellow	LCS, NIAAA
F. Romero	Graduate Student	UNM

Objectives:

This is a study on alcoholism in families derived from three American Indian populations. The objectives are 1) to characterize the clinical phenotype of alcoholism, including psychiatric comorbidity, 2) to ascertain the extent to which alcoholism in these Indian groups is familial, and 3) to locate using dispersed and candidate genetic markers genes responsible for alcoholism risk. The candidate loci would include genes connected to serotonin function and ethanol metabolism as well as markers for alcoholism defined in other populations.

Methods employed:

Familial alcoholics and their families are clinically assessed in collaboration with Dr. Gerald L. Brown (Unit on Family Studies, LCS, NIAAA). Family members receive a comprehensive clinical psychiatric and psychological evaluation, including the SADS-L psychiatric interview, life history of impulsive behavior, the MAST for alcohol and drug use, a structured family history and several psychological tests including the MMPI and EPQ (Eysenck Personality Questionnaire). Consensus clinical diagnoses are made by two raters blind to subject identities and incorporating data from corroborative family interviews, the MAST and other records.

For DNA and protein genetic markers, a blood sample is obtained and a lymphoblastoid cell line is established. Cells are cultivated to provide sufficient DNA and protein for marker studies and for cryopreservation.

For genetic linkage, we test for nonrandom assortment of marker and phenotype in families, linkage existing if the two are transmitted as if coupled together or as if in repulsion. Linkage is also sought by searching for associations in populations. The likelihood of establishing linkage depends in part on the number of genetic markers typed. In collaboration with Stephen O'Brien (NCI, Laboratory of Viral Carcinogenesis) and Michael Dean (Program Resources Inc, Frederick, MD) we are typing 200 DNA polymorphisms.

Major Findings:

Preliminary studies on families collected in Bethesda revealed considerable assortative mating and a high density of alcoholism in affected pedigrees. Therefore, for familial genetic analyses directed towards genetic linkage, we have attempted to minimize heterogeneity by collecting American Indian families and Finnish impulsive/aggressive families and by following EEG and ERP variants in families.

Alcoholism and its consequences are severe societal problems in American Indians but are found nonuniformly across different tribes. One goal of these studies is to address the genetic epidemiology of alcoholism in diverse American Indian groups using a structured family history and a psychiatric interview. To date, we have evaluated more than 200 Cheyenne Indians, over 100 Jemez Pueblo Indians and a small group of Pima Indians. From both tribes, several large families have been studied and a lymphoblastoid cell line has been established for each individual in the study. A salient clinical finding is a high rate of psychiatric comorbidity, particularly anxiety disorders, in American Indian alcoholics.

A large panel of genetic markers has been typed in Cheyenne alcoholics and controls. We are currently performing analyses to detect evidence for genetic linkage.

In collaboration with the NIH diabetes epidemiology group (Drs. W. Knowler and P. Bennett), we recently initiated a study of the Pima Indians at the Gila River Reservation. At this reservation, psychiatric interviewers have been trained. A large cooperative family has been identified and a detailed pedigree has been constructed for that family with the aid of elderly informants, and lymphocytes from 15 subjects within that family have been collected.

Significance to Biomedical Research and the Program of the Institute:

Familial linkage studies offer a direct method for bridging the gap between fundamental molecular mechanisms and behavioral phenotypes related to the vulnerability to alcoholism. Genes for alcoholism will be hard to map in Caucasian families because alcoholism is clinically (and probably genetically) heterogeneous, environmentally influenced, highly prevalent and marked by assortative mating. The greater environmental and genetic homogeneity of these Indian communities greatly increases the likelihood of mapping genetic loci or identifying factors predisposing individuals to alcoholism. The families and populations detected here will also provide useful datasets for testing the generalizability of genetic linkages found in other populations. For many Indian communities, alcohol has a tragic impact. Alcoholism accounts for more than 50% of deaths in the three Indian communities in our study and it degrades the quality of family and social relationships at many levels. Careful, structured, in depth evaluations will enable us to accurately compare the clinical phenomenology of alcoholism in these Indian populations with each other and to Caucasians. By defining age of onset and severity, by accurately defining psychiatric comorbidity, and by following the transmission of pathologies in families, we can gain clues as to the causes of alcoholism.

Proposed course:

We will complete the blind-rating for psychiatric diagnoses from the Cheyenne and Jemez Pueblo families. This will enable us to complete analyses for genetic linkage and clinical genetic associations to alcoholism in these groups. The family cell line and psychiatric interview data collection for the Pima Indians will be completed this year and this data will be used in transmission and linkage analyses. If tribal approvals can be obtained, we will also collect random adult population samples from the three Indian communities to enable us to make more powerful interpretations concerning the genetic epidemiology and associations of psychiatric traits and alcoholism in Indians.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00282-02 LCS

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Molecular Genetic Studies on the Dopamine D2 Receptor

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	D. Goldman	Section Chief	LCS, NIAAA
Others:	A. Bolos	Visiting Fellow	LCS, NIAAA
	G. Brown	Unit Chief	LCS, NIAAA
	M. Linnoila	Scientific Director	NIAAA

COOPERATING UNITS *(if any)*
Program Resources Inc., Frederick, MD (M. Dean);
Department of Psychiatry, University of Helsinki (M. Virkkunen)

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
10	6	4

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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A highly significant population genetic association was reported between alcoholism and a TaqI DNA polymorphism recognized by the dopamine D2 receptor gene probe. We tested this linkage using two D2 DNA markers in a population of well-characterized alcoholics versus population controls and in two informative pedigrees. The second DNA marker was a novel one, generated by the technique of polymerase chain reaction (PCR) followed by nondenaturing DNA electrophoresis of single-stranded DNA to detect a conformational polymorphism (SSCP). The dopamine D2 receptor gene and alcoholism were shown to be unlinked in the general Caucasian population. There was also no association between the dopamine D2 receptor polymorphism and alcoholism in a population of impulsive alcoholic Finns. In that population, we were also able to show that there was no relationship between D2 marker status and an indicator of central dopaminergic function: CSF homovanillic acid (HVA) level.

Project Description:

Investigators:

D. Goldman	Section Chief	LCS, NIAAA
A. Bolos	Visiting Fellow	LCS, NIAAA
G. Brown	Unit Chief	LCS, NIAAA
M. Dean	Staff Scientist	PRI, Frederick, MD
M. Linnoila	Scientific Director	NIAAA
M. Virkkunen	Senior Lecturer	Univ. of Helsinki Finland

Objectives:

A highly significant population genetic association had been reported between alcoholism and a polymorphism recognized by a dopamine D2 receptor gene probe. We sought to confirm this genetic linkage.

Methods employed:

Linkage studies in human families

Forty Caucasian alcoholics and all members of two informative families were clinically assessed in collaboration with Dr. Gerald L. Brown (Unit on Family Studies, LCS, NIAAA). Subjects received a comprehensive clinical psychiatric and psychological evaluation, including the SADS-L psychiatric interview, life history of impulsive behavior, the MAST for alcohol and drug use, a structured family history and several psychological tests including the MMPI and EPQ (Eysenck Personality Questionnaire). Consensus clinical diagnoses were made by two raters blind to subject identities and incorporating data from corroborative family interviews and from other records. Alcoholics were subtyped according to comorbidity, severity and age of onset of drinking problems. In Finland, a group of impulsive alcoholics and Finnish population controls were diagnosed according to DSMIII-R criteria. Homovanillic acid (HVA) and other monoamines and monoamine metabolites were measured in the CSF by HPLC with electrochemical detection.

For DNA and protein genetic markers, a blood sample was obtained and a lymphoblastoid cell line was established. Marker frequencies in controls were established in a population of more than 100 Caucasians. Cells were cultivated to provide sufficient DNA and protein for marker studies and cryopreservation.

For genetic linkage, we tested for a population association and for nonrandom assortment of marker and phenotype in two families in which there was an alcoholic parent with the A1 variant found by Blum et al. to be associated with alcoholism.

For the TaqI dopamine D2 RFLP, the genomic probe used by Blum et al. was obtained from D. Grandy and used in Southern hybridization analysis.

For the polymorphism detected by PCR-SSCP (polymerase chain reaction-single strand conformational polymorphism), a fragment of the 3' noncoding region of the gene was amplified by the polymerase chain reaction. This fragment was electrophoresed in its single stranded form in a nondenaturing gel, revealing a polymorphism. In the PCR-SSCP technique, polymorphisms result from sequence variants that alter the secondary structure of the DNA fragment.

Major findings:

In the population study of 40 alcoholics and over 100 controls, no association was observed between either dopamine D2 polymorphism and alcoholism, early or late onset alcoholism, severe or less severe alcoholism or alcoholism accompanied or unaccompanied by comorbidity.

For the dopamine D2 TaqI RFLP, a third rare allele was described. Because it has been asserted that the D2 allele is related to severe alcoholism and to dopamine function, we recently analyzed the D2 receptor in a population of severe Finnish alcoholics and population controls. That group had also had CSF homovanillic acid (HVA) measured. HVA is a dopamine metabolite whose level is an index of brain dopamine turnover. There was no correlation between D2 type and HVA level and no relationship to diagnosis in this extreme alcoholic population.

Significance to Biomedical Research and the Program of the Institute:

Our inability to replicate the strong dopamine D2 population linkage described by Blum et al. in a population of well-characterized alcoholics and in Finnish alcoholics in whom the dopamine metabolite HVA was measured reemphasizes the concept that, in Caucasians, alcoholism is likely to be a genetically heterogeneous disorder that is not in strong genetic linkage disequilibrium with a marker at a single locus.

Discovery of the dopamine D2 SSCP polymorphism provides a linkage marker for genetic studies on behavioral traits and for determining the chromosomal location of the dopamine D2 genes.

Proposed Course:

We will perform genetic linkage analyses using the CEPH collection of pedigrees to determine the relationship of the two available D2 polymorphisms to the different dopamine D2 loci.

Publications:

Bolos AM, Dean M, Lucas-Derse S, Ramsburg M, Brown GL and Goldman D. Population and pedigree studies reveal a lack of association between the D2 receptor gene and alcoholism, JAMA 1990;264:3156-60.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AA 00283-02 LCS

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Molecular Genetic Studies on Enzymes of Alcohol Metabolism

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	D. Goldman	Section Chief	LCS, NIAAA
Others:	T. Moretti	IRTA Fellow	LCS, NIAAA

COOPERATING UNITS *(if any)* ~~Lab. of Viral Carcinogenesis, NCI (S. O'Brien); Program Resources~~ Inc., Frederick, MD (M. Dean); Univ. of Indiana School of Med. (H. Edenberg); Charing Cross & Westminster Med. Sch., London (E. Joyce); Vanderbilt U. Sch. of Med. (P. Martin, B. Singleton); Lab. of Molec. Microbiology, NIAID (C. Kozak)

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NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1	0	1

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  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Among the candidate genetic loci for differences in alcohol's toxic effects are genes encoding alcohol metabolic enzymes and thiamine-dependent enzymes including transketolase. Class III ADH (ADH5) is an unusual ADH which has an important role in the metabolism of formaldehyde (Koivusalo, 1990). This enzyme could thus play a role in methanol toxicity and in the metabolism of methanol found as a trace contaminant in alcoholic beverages. We have shown that this is the only ADH present in significant amounts in brain. We previously cloned the human class III alcohol dehydrogenase, characterized its sequence and expression and discovered a moderately informative human Sac I RFLP. We have located the ADH5 gene to the same chromosomal region in mouse and man, approximately 5 cM from the other ADH genes. We have also located several ADH pseudogenes. Utilizing the Sac I RFLP and a second more informative polymorphism identified by the PCR-SSCP method, we are investigating whether this locus is associated with the Wernicke-Korsakoff syndrome. Collaboratively with Drs. Martin and Singleton, who have cloned transketolase, we have identified a polymorphism for this gene and mapped it by genetic linkage analysis and are investigating whether it is associated with the Wernicke-Korsakoff syndrome.

Project Description:

Investigators:

D. Goldman	Section Chief	LCS, NIAAA
M. Dean	Staff Scientist	PRI, Frederick, MD
H. Edenberg		University of Indiana Medical School
E. Joyce		Charing Cross, London
C. Kozak	Research Biologist	LMM, NIAID
P. Martin	Associate Professor	Vanderbilt University School of Medicine
T. Moretti	IRTA Fellow	LCS, NIAAA
S. O'Brien	Chief	LVC, NCI
B. Singleton		Vanderbilt University School of Medicine

Objectives:

The main objectives are 1) to clone and study loci (candidate genes) involved in ethanol metabolism, identifying polymorphisms of these loci for use as linkage markers and mapping the genes; 2) to establish genetic linkage between candidate gene markers and the vulnerability to alcohol or alcohol-induced damage; and 3) ultimately to identify molecular variants underlying differences in alcohol response.

Methods:

For molecular cloning, we prepared cDNA and genomic DNA libraries and screened these for relevant cDNA clones using oligonucleotide probes. For class III ADH, the protein was purified, peptides were sequenced and corresponding oligonucleotides were synthesized. The human transketolase cDNA was cloned and sequenced by Dr. Singleton (Vanderbilt University).

For expression studies, mRNA was extracted from tissues, at times enriched for polyA+ mRNA, electrophoresed on agarose gels, transferred to membrane and then hybridized with the specific radiolabelled probe.

For genomic mapping and linkage studies, genetic variants were detected by restriction enzyme digestion, Southern transfer and use of the specific probe. Additional variants were detected by PCR-SSCP. In PCR-SSCP, a specific region of the DNA is amplified by the polymerase chain reaction and then single strand conformational polymorphisms are electrophoretically detected by the differential mobility of DNA strands which have undergone substitutions. Mapping was accomplished using either the linkage method or by analysis of panels of hybrid cell lines containing different combinations of human chromosomes.

Major Findings:

ADH5: Cloning and comparative mapping of human class III ADH

With the cloning of the ADH5 cDNA, DNA probes for all of the known human alcohol dehydrogenases are now available. This has allowed new advances to be made in the understanding of the structural organization and functional enzymology of these genes. The five known human alcohol dehydrogenases (ADHs) which had been mapped to chromosome 4 included three class I ADHs and one class II gene, all of which metabolize ethanol, and the unusual class III ADH, which does not. Koivusalo et al. showed that one important function of class III ADH is in one carbon metabolism: the dehydrogenation of formaldehyde to formic acid. This is an important reaction in the mediation of methanol toxicity and in the metabolism of methanol found in alcoholic beverages.

We isolated a full-length human class III cDNA (ADH5) and used it to define the sequence, homology, expression and chromosomal localization of ADH5 and to localize a homologous mouse gene. The nearly full-length 1613 nucleotide cDNA contained 58 nucleotides of 5' nontranslated sequence, a complete coding sequence of 1122 bases and 433 of 3' nontranslated sequence. In their coding sequences, the three major ADH classes were similar with the class II and III ADHs sharing the highest sequence identity (67%). In contrast to other ADHs whose expression is restricted, class III ADH was expressed ubiquitously in human and rodent tissues. A polymorphism was recognized by the ADH5 cDNA probe after Sac I digestion of human genomic DNA. Polymorphic fragments were 7.3 and 6.1 kb in size and the allele frequencies were 0.83 and 0.17, respectively. An additional PCR SSCP polymorphism was discovered by analysis of intron sequences. For finer resolution mapping, these polymorphisms were typed across the entire collection of CEPH families, and ADH5 was found to be located approximately 5 cM from the class I ADHs. A class III-like ADH was mapped to mouse chromosome 3, also approximately 5 cM from the ADH gene complex. Collaboratively with Dr. Edenberg and colleagues (Indiana University School of Medicine) the mouse class III ADH gene was cloned and sequenced. Tamyra Moretti has recently identified additional class III ADH pseudogenes in both human and mouse.

Use of human ADH5 and transketolase probes in studies on alcohol-induced toxicity

The causes of individual variability in ethanol-induced damage to the brain and to other organs are unknown. Only a fraction of alcoholics suffer from alcoholic brain disease including Wernicke-Korsakoff syndrome. Genetic association and direct candidate gene analyses (for example, gene sequencing) may shed light on the inherited predisposition. Some evidence suggests that a variant of transketolase is responsible for the vulnerability to Wernicke-Korsakoff disease, however the issue is controversial. In a collaborative study with Drs. Singleton and Martin (Vanderbilt University School of Medicine) and Dr. Joyce (formerly with NIAAA), we will establish whether a specific association between Wernicke-Korsakoff's Disease and transketolase exists. Using the human transketolase cDNA cloned by Drs. Singleton and Martin and the polymorphisms we have discovered, we have undertaken a population association study with Dr. Joyce. Because of the possible role of methanol toxicity in inducing brain damage, we are also using the human ADH5 probe, sequence and polymorphisms in this study.

Significance to Biomedical Research and the Program of the Institute

The alcohol dehydrogenases are the primary enzymes responsible for the metabolism of ethanol and class III ADH has an important role in the metabolism of formaldehyde and thus plays a role in methanol toxicity and in the metabolism of methanol found in alcoholic beverages. With our cloning of the class III alcohol dehydrogenase cDNA, DNA probes for all of the known human alcohol dehydrogenases are now available. This will allow new advances to be made in the understanding of the structural organization, functional enzymology and control of expression of these genes. For example, our results have revealed that this gene is structurally highly distinct, constitutively transcribed in most or all tissues and in fetal life and present in an extended gene complex with the other ADHs in both mouse and man.

Clive Harper's neuropathological surveys in Australia and elsewhere have revealed that Korsakoff's lesions are present in several percent of brains from random hospital autopsy series. The transketolase genetic vulnerability hypothesis remains our best explanation for explaining the differential vulnerability to Wernicke-Korsakoff's disease and ADH5 is also a logical candidate gene for differential vulnerability to alcohol-induced brain damage.

Proposed Course:

The human ADH5 and transketolase DNA polymorphisms are being used in linkage analyses aimed at understanding alcohol-induced toxicity.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00234-09 LCS

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Molecular Studies on Tryptophan Hydroxylase Gene Expression

PRINCIPAL INVESTIGATOR *(List other professionel personnel below the Principel Investigator.) (Neme, title, leboratory, end institute effiliation)*

PI:	D. Nielsen	Senior Staff Fellow	LCS, NIAAA
Others:	L. Akhtar	Chemist	LCS, NIAAA
	M. Filling-Katz	Senior Staff Fellow	LCS, NIAAA
	B. Giblin	NRC Fellow	LCS, NIAAA
	D. Goldman	Section Chief	LCS, NIAAA
	M. Linnoila	Scientific Director	NIAAA
	J. Raskin	IRTA Fellow	LCS, NIAAA

COOPERATING UNITS *(if any)*
Institute for Behaviorial Genetics, University of Colorado, Boulder (T. Johnson); Program Resources Inc. (M. Dean); University of Helsinki (M. Virkkunen); Clemson University (E. Pivorun); Laboratory of Molecular Microbiology, NIAID (C. Kozak)

LAB/BRANCH
Laboratory of Clinical Studies

SECTION
Section on Genetic Studies

INSTITUTE AND LOCATION
NIAAA, 9000 Rockville Pike, Bethesda MD 20892

TOTAL MAN-YEARS: 10	PROFESSIONAL: 6	OTHER: 4

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use stendard unreduced type. Do not exceed the spece provided.)*

In order to identify genetic determinants of alcoholism we have focused on human and murine behaviors that are associated with serotonin function. Serotonin synthesis is controlled by the activity of the enzyme tryptophan hydroxylase (TrH). TrH is the rate-limiting enzyme in the production of this neurotransmitter. Therefore, we hypothesize that control of the TrH gene expression and genetic variants of TrH play a major role in the etiology of serotonin-associated behaviors. We have cloned and sequenced the cDNA and gene coding for murine TrH. The TrH gene was located to chromosome 7. Cloning and sequencing of this gene also allowed the characterization of its putative regulatory region as well as its intron/exon boundaries. We are extending these studies to investigate the factors and DNA sequences controlling the expression of this gene. Allelic variants of the TrH gene have been identified by SSCP analysis in both humans and mice. Utilizing the CEPH families, we are mapping the tryptophan hydroxylase gene in the human genome. We are studying the relationship of this polymorphism to impulsive behavior in impulsive and alcoholic Finns. In recombinant LSXSS inbred strains of mice, alcohol induced sleep time showed a significant association with the TrH DNA polymorphism. DNA constructions are being produced that will be inserted into the genome of mice. These studies and those on natural genetic variants of TrH will be used to quantitate the serotonergic component of behavior as well as to determine the DNA sequences required for tissue specific gene expression.

Previous title: Molecular Studies of Serotonin Genes

Project Description:

Investigators:

D. Nielsen	Senior Staff Fellow	LCS, NIAAA
L. Akhtar	Chemist	LCS, NIAAA
M. Dean	Staff Scientist	PRI, Frederick, MD
M. Filling-Katz	Senior Staff Fellow	LCS, NIAAA
B. Giblin	NRC Fellow	LCS, NIAAA
D. Goldman	Section Chief	LCS, NIAAA
T. Johnson	Professor	IBG, UC, Boulder, CO
C. Kozak	Research Biologist	LMM, NIAID
M. Linnoila	Scientific Director	NIAAA
J. Raskin	Graduate Student	LCS, NIAAA
M. Virkkunen	Senior Lecturer	Univ. of Helsinki, Finland

Objectives:

A main objective of the Section on Genetic Studies is to understand the genetic determinants of serotonin function. These studies are aimed at identifying the genetic loci involved in the clinical subtype of alcoholism distinguished by impulsive behavior. Low serotonin turnover has been strongly implicated in human impulsive and aggressive behaviors and with alcohol preference in rodents. We are focusing on the tryptophan hydroxylase gene since it codes for the rate-limiting enzyme in the production of serotonin. The regulation of this gene's expression and its role in the control of behaviors in humans and mice are being investigated.

Methods employed:

Genetic cloning and gene constructions were performed by standard procedures. Sequences from mouse tryptophan hydroxylase, human metallothionein, firefly luciferase, HSV thymidine kinase, Tn5 neomycin-kanamycin resistance, chloramphenicol acetyltransferase genes as well as SV40, RSV and MMTV promoters and regulatory elements, and pUC sequences have been combined to form the appropriate vectors for the studies outlined below. Methods used include restriction endonuclease cutting, filling with Klenow enzyme, fragment isolation, ligation, site-specific mutagenesis and dideoxy sequencing.

Serotonin synthesizing and secreting cell lines are grown in tissue culture. Cell lines used include TT cells, a human medullary thyroid carcinoma cell line, 1C11 (I-1069) cells, a mouse neuronal precursor cell line and P815 cells, a mouse mastocytoma cell line. Mouse 3T3 fibroblast cells will serve as a nonserotonergic control cell line. These cell lines will be transfected with the appropriate DNA plasmid constructions by electroporation. Transiently transfected cells as well as stable, G418 resistant, cell lines will be analysed. Gene function will be assayed through the use of the highly sensitive reporter genes luciferase and chloramphenicol acetyltransferase as well as by mRNA dot spot and Northern analysis.

For transfection and expression studies, mRNA was extracted from cell lines and tissues and at times was enriched for polyA+ mRNA. The RNA was either electrophoresed and transferred to nylon membranes or spotted directly onto the membranes, and hybridized with the specific radiolabelled probe.

Several DNA constructions will be introduced into transgenic mice through the services of the National Transgenic Development Facility operated by DNX. The effects of these fusion genes on behavior will be quantified using behavioral tests.

Single strand conformation polymorphism (SSCP) were performed on DNA samples of human and mice. Several regions of the tryptophan hydroxylase gene were amplified with primers by polymerase chain reaction, denatured and ran on nondenaturing polyacrylamide gels. Bands were analyzed by autoradiography or phosphor imaging.

Major Findings:

Serotonin is synthesized in the brain in the raphe nuclei. It is also made in the gut and blood platelets, and in the pineal gland where it is rapidly metabolized to melatonin. Tryptophan hydroxylase is only found in cells that produce serotonin. In the brain, tryptophan hydroxylase (TrH) is the rate-limiting enzyme in the production of serotonin. The Km of TrH has been reported to be 50-120 μM. Tryptophan, the substrate of this reaction, is present in the brain at nonsaturating levels of 30 μM. The product of this hydroxylation, 5-hydroxytryptophan, is rapidly decarboxylated to serotonin (5-hydroxytryptamine). Hence, the concentration of TrH, as well as its activity, regulate the production of serotonin in the brain. The concentration of TrH is regulated by its synthesis from mRNA which is directly related to the level of TrH gene activity.

TrH is a member of the family of aromatic amino acid monooxygenases which include tyrosine hydroxylase and phenylalanine hydroxylase. A full length, murine TrH cDNA was cloned from a mastocytoma cDNA library. This sequence encodes a 447 amino acid protein that is 3 amino acids longer than the rabbit pineal TrH due to a small duplication near the amino terminus. The mouse tryptophan hydroxylase shares 88% similarity with rabbit pineal TrH and is approximately 50% identical in sequence to the other monooxygenases. Identities were lower in the 5' amino terminal coding region as this region codes for the noncatalytic domain of TrH. Portions of the TrH cDNA were expressed in E. coli as fusion proteins which were immunological cross-reactive and enzymatically active. This enabled us to map the region of the primary structure containing the catalytic domain. Northern analysis of mRNA isolated from various mastocytoma cell lines, pineal, duodenum and midbrain revealed two TrH mRNA size classes. One corresponded to the full length 1900 bp TrH mRNA while the larger 4000 bp mRNA, which represented only 5-10 % of the total TrH mRNA, is presumably a partially processed, precursor TrH mRNA.

The TrH cDNA was used to probe a mouse genomic library. Overlapping clones spanning 40 kb of the murine gene were isolated. The murine TrH gene is 21 kb in length and, except for the 5' end of the gene, its intron/exon borders were highly conserved compared to tyrosine hydroxylase and phenylalanine hydroxylase. In collaboration with C. Kozak (NIAID), the murine TrH gene was mapped to mouse chromosome 7. Sequence analysis of the 5' upstream regulatory regions reveal the presence of many putative regulatory regions. Besides well conserved TATA and CAAT boxes are sequences that are related to the consensus binding sequences of AP-1, AP-2 and AP-3. Regulatory sites are present for cyclic AMP regulation, the urea cycle enzyme sequence, retinoblastoma gene product control element, Sp1, NF-1 and octomer binding sites as well as general helix-turn-helix protein binding sites. To analyze this plethora of presumptive regulatory sites we have fused the 5' upstream region of the mouse TrH genomic clone to the gene coding for firefly luciferase. When transfected into tissue culture cells a fusion mRNA will be produced containing 106 bases of the 5' untranslated region from TrH followed by 36 bases of luciferase 5 untranslated region. Translation will begin with the luciferase AUG. This fusion allows gene expression to be easily assayed with extreme sensitivity. This DNA will be introduced into various tissue culture cells by electroporation along with a control plasmid (either RSV-CAT or SV40-CAT) to provide an unregulated, internal tranfection efficiency control. The DNAs will be transfected into several serotonin synthesizing cell lines: TT cells, a human medullary thyroid carcinoma cell line, 1C11 (I-1069) cells, a mouse neuronal precursor cell line and P815 cells, a mouse mastocytoma cell line. Mouse 3T3 fibroblast cells will be transfected as a nonserotonergic control cell line. Compounds including serotonin, 5-HIAA, dibuteryl cAMP, tryptophan, dexamethasone, reserpine, L-kynurenine and p-chlorophenyalanine will be assayed to measure their effect on transcription. Ones found to regulate TrH gene expression will be analyzed in all the cell lines to determine tissue-specific effects. To analyze DNA sequences which may control expression, deletions of the TrH promoter are being constructed by deleting to convenient restriction endonuclease sites and by site-directed mutagenesis. This will allow the mapping of regulatory sequences at which drugs act as well as sites determining tissue specific expression.

To find a polymorphism in the TrH gene in humans we amplified human DNA using the single strand confirmation polymorphism (SSCP) technique. A polymorphism was found in intron 7 of the human tryptophan hydroxylase gene. In collaboration with Mike Dean, DNA samples from the CEPH families were typed to determine population allele frequency and genetic linkage map location. Allele frequencies in Caucasians were 0.38 and 0.62 and the gene will be precisely mapped in the human genome. In addition collection of impulsive and alcoholic Finns is being analyzed and the distribution of genotypes compared to their psychologically defined behaviors and to levels of CSF 5-HIAA (a serotonin metabolite which indicates brain serotonin turnover). A number of these subjects have very low CSF 5-HIAA levels. Preliminary results from a subgroup of subjects indicate a significant correlation between impulsive behavior and genotype. Therefore we are expanding this analysis to the entire Finnish dataset.

An SSCP polymorphism has also been identified in mice in intron 8 of the TrH gene. In collaboration with Tom Johnson at the U. of Colorado, the genotypes of LSXSS recombinant inbred mouse strains of mice were determined. All but one of the alcohol sensitive (long sleep, LS) strains had the same genotype. The alcohol insensitive (short sleeping, SS) strains were varied in genotype. This polymorphic region is now being sequenced. A TrH polymorphism will also be used to examine deermice (Peromyscus maniculatus) with Bridget Giblin and in collaboration with Edward Pivorun (Clemson University). Dr. Pivorun has shown that deermice exhibit population genetic variation in their ability to enter torpor. Considerable evidence exists that torpor may be a trait related to serotonin turnover. Therefore, TrH gene function and genetic variation may have a major influence on this behavior.

To ascertain the function of TrH in whole animals, transgenic mice will be produced. Several DNA constructs are being made which will be introduced into mice. The effects of these on behavior will be assessed. The questions to be addressed are what is the effect of having abnormally high or low levels of TrH in the brain. DNA constructions are being made that will allow the regulated switching on and off of TrH expression. We will also attempt to create a mouse strain that will lack serotonin producing cells. This mouse strain also will allow us to confirm the sequences necessary for tissue specific expression directed by the TrH promoter through the use of in vitro hybridization.

Significance to Biomedical Research and the Program of the Institute:

Tryptophan hydroxylase is the rate-limiting enzyme in the production of serotonin. Low turnover of serotonin is associated with impulsive and aggressive behaviors, with increased behavioral arousal and with intolerance to delay. These behaviors are prominent features of early onset alcoholism associated with features of antisocial behavior. It is therefore likely that the level and activity of tryptophan hydroxylase plays a major role in these behaviors. Our studies on the tryptophan hydroxylase gene has improved our understanding of the structure, function and expression of this enzyme. We now have the whole gene cloned so that we can elucidate the mechanisms governing its expression. Furthermore, polymorphic alleles may identify a genetic linkage that will reveal the cause of several genetic behavioral differences in humans and mice.

Proposed Course:

To understand the effects of environmental and physiological factors such as stress on serotonin function and impulsive behavior we are investigating the regulatory control of tryptophan hydroxylase. Using in vitro transfection of tissue culture cells we are delineating the factors controlling TrH gene expression.

The studies will reveal sequences in the TrH promoter involved in this regulation as well as those determining tissue specificity. Once these controlling sites are identified we will able characterize and identify the factors mitigating these actions. The TrH polymorphisms we have identified in both humans and mice are being characterized further. When the TrH gene is firmly linked to their cognate behaviors, the TrH genes will be isolated. These polymorphic TrH genes will be analyzed to determine how they vary in function and how this controls behavior. Transgenic mice are to be produced to provide a whole animal model of serotonergic functioning. Behavior can then be related directly to TrH gene and serotonergic activity.

Publications:

Nielsen DA, Shapiro DJ. Estradiol and Estrogen Receptor-Dependent Stabilization of a Minivitellogenin mRNA Lacking 5,100 Nucleotides of Coding Sequence, Mol Cell Biol 1990;10:371-6.

Nielsen DA, Shapiro DJ. Insights into hormonal regulation of mRNA stability, Mol Endo 1990;4:953-7.

Stoll J, Goldman D. Isolation and structural characterization of the murine tryptophan hydroxylase gene, J Neuroscience Research 1991;28:457-65.

Stoll J, Kozak CA, Goldman D. Characterization and chromosomal mapping of a cDNA encoding tryptophan hydroxylase from a mouse mastocytoma cell line, Genomics 1990;7:88-96.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00239-08 LCS

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Alcoholism-Associated Cognitive Impairment and Organic Brain Syndrome

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	M. Eckardt	Section Chief	LCS, NIAAA
Others:	R. Lister	Visiting Scientist	LCS, NIAAA
	R. Rawlings	Mathematical Statistician	LCS, NIAAA

COOPERATING UNITS *(if any)*

Laboratory of Clinical Physiology, NIA (H. Weingartner)

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section on Clinical Brain Research

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose of this study is to examine the neuropsychological performance of several clinically defined populations of detoxified male alcoholics. Comparisons will be made among detoxified alcoholics with clinically defined chronic organic brain syndromes, dementia or amnestic syndrome, less cognitively impaired alcoholics who are in alcoholism treatment programs; and nonalcoholic controls.

PROJECT DESCRIPTION

Investigators:

M. Eckardt	Section Chief	LCS, NIAAA
R. Lister	Visiting Scientist	LCS, NIAAA
R. Rawlings	Mathematical Statistician	LCS, NIAAA
H. Weingartner	Section Chief	LPC, NIA

Objectives:

Chronic alcohol abuse may lead to two clinically and neuropathologically distinguishable syndromes: alcoholic dementia and alcoholic amnestic syndrome (called Korsakoff's psychosis), which together constitute the second most common cause of dementia in adults (approximately 10%). These two alcohol-related organic brain syndromes may represent the extremes on a cognitive dysfunction scale with alcoholic dementia characterized by a global intellectual decline, whereas alcoholic amnestic syndrome can be characterized as a severe and persistent amnesia with a relative sparing of other intellectual functions. The majority of alcoholic patients in clinical practice fall somewhere in between. In the present study, we propose to use a comprehensive battery of neuropsychological tests to differentiate alcoholic dementia from alcoholic amnestic syndromes. Less cognitively impaired alcoholics and normal, age-matched volunteers will be evaluated similarly. Comparisons among these groups will lead to a better characterization of cognitive similarities and differences in these groups.

Methods Employed:

Two clinically defined groups of alcoholics will be evaluated: participants in an alcoholism treatment program and those with sufficient, clinically defined, cognitive impairment so as to be judged as not likely to benefit from the treatment program. The latter group will be separated by neuropsychological performance into those with alcoholic dementia and those with alcoholic amnestic syndrome.

It has been shown previously that the neuropsychological performance of neurologically impaired alcoholics with dementia can be differentiated from that of alcoholics with amnestic syndrome. We plan to use a more comprehensive and sensitive test battery to better understand this differentiation.

The neuropsychological test battery is designed to obtain a global assessment of cognitive skills, an in-depth examination of memory functions, and an assessment of alcoholism-related cognitive decrements. The examination will take about 12 hours to complete. The battery consists of Halstead-Reitan Battery including Trails A and B; Wechsler Adult Intelligence Scale; Wechsler Memory Scale; Wisconsin Card Sorting Test; and selected memory tests designed to compare episodic versus semantic learning, automatic versus effortful learning, and language versus non-language learning.

Data will also be collected on socioeconomic status, personality, childhood history of attention deficit disorder and hyperactivity, and drug use history, including alcohol.

Major Findings:

We are still collecting data and have begun to analyze it.

Significance to Biomedical Research and the Program of the Institute:

It has been well documented that alcoholics have impaired brain function. The present study is designed to better characterize the cognitive deficits observed in alcoholics so that more appropriate pharmacological intervention will be possible.

Proposed Course:

Data collection and analysis are continuing.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00240-12 LCS

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Cognitive Function in Male Alcoholics

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	M. Eckardt	Section Chief	LCS, NIAAA
Others:	R. Rawlings	Mathematical Statistician	LCS, NIAAA

COOPERATING UNITS *(if any)*

Department of Psychiatry and Human Behavior, Univ. of California, Irvine, CA (L. Gottschalk); TRISARD, Bethesda, Naval Hospital, Bethesda, MD (D. Groden); Neuroscience Research Branch, NIDA (J. Stapleton)

LAB/BRANCH

Laboratory of Clinical Studies

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NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	0.5	1.0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This series of studies is concerned with cognitive function in detoxified male alcoholics. Recent and chronic alcohol consumption variables were found to interact with each other and with age and education in a non-linear fashion in predicting neuropsychological performance. Increased consumption predicted decreased performance, even on tests whose mean scores were in the normal range. Little or no improvement in performance was demonstrable with short-term abstinence (14-20 days), although long-term abstinence (7 months) was associated with improvement. Similarly, hepatic and hematologic characteristics of long-term abstainers improved, whereas these systems functioned abnormally in people who continued to consume alcoholic beverages, albeit at significantly reduced levels. Relationships between various pretreatment prediction variables and subsequent outcome are also being studied. Increased risk of relapse was associated with excessive drinkers who were relatively early in their alcoholic careers as assessed by years of abusive drinking and accumulated lifetime exposure to alcohol. Although statistically significant relationships were observed between scores on certain neuropsychological tests and posttreatment alcohol consumption, neuropsychological evaluation was determined to be of limited clinical utility.

Project Description

Investigators:

M. Eckardt	Section Chief	LCS, NIAAA
L. Gottschalk	Professor	Univ. of CA, Irvine, CA
D. Groden	Chief	TRISARD, BNH
R. Rawlings	Mathematical Statistician	LCS, NIAAA
J. Stapleton	Staff Fellow	NR, NIDA Hospital

Objectives:

The present series of studies was designed to document the presence of CNS impairment in male alcoholics, discern possible etiological factors related to this impairment, and determine whether improvement in function is associated with subsequent abstinence. Additional questions concern whether treatment should commence immediately after detoxification, relationships between CNS function and treatment outcome, and neuropsychological consequences of post-treatment alcohol consumption.

Methods Employed:

A battery of 24 neuropsychological tests was administered to drug-free alcoholic inpatients (n=91) within 7 days of their last drink and again 17 days later. To control for practice effects, a nonalcoholic medical control group (n=20) also took the test battery twice, with approximately the same interval elapsing between administrations. Another group of alcoholic inpatients (n=32) took the tests only once, 14-31 days after their last drink. After patients completed the 21-day treatment program, they were contacted on a monthly basis to determine drinking behavior. At the end of 7 months, they returned to the hospital. Before testing, a breathalyzer and/or clinical laboratory determination of blood alcohol level was carried out in an attempt to ensure sobriety during testing. The 24 cognitive tests were then administered in a random order. Self administered questionnaires were used to calculate post-treatment frequency of drinking alcohol and quantity consumed per occasion. Patient-supplied collaterals were then contacted to verify the patients' self-reports. Approximately 24 months after entrance into the treatment program, 17 of the original 91 patients were located and agreed to take the entire battery of neuropsychological tests again.

Major Findings:

Cognitive performance in drug-free alcoholic men is significantly predicted by chronic and recent drinking practices. Furthermore, it appears that certain patterns of consumption may accelerate the alcohol-induced decline of brain function. Little or no improvement in cognitive performance was demonstrable with short-term abstinence when controls were included for the effects of repeated testing. Continued alcohol consumption by recovering alcoholics is associated with reduced cognitive performance, while those who abstained have improved test scores. Neuropsychological performances determined 24 months after entrance into the program were at the same levels as at 7 months after entrance. Similar findings were observed in clinical laboratory tests, with long-term abstainers (7 months) having improved hepatic and hematologic functioning in contrast to the continued abnormal functioning observed in those people who continued to drink, albeit at significantly reduced levels. Further analysis of these clinical laboratory tests revealed widespread and persistent alcoholism-related alterations in organ system functioning, even after long-term abstinence (7 or 24 months).

Male alcoholics' pre-treatment levels of alcohol consumption were found to be related statistically to post-treatment levels of consumption with an increased risk of relapse associated with excessive drinkers who were relatively early in their alcoholic careers, as assessed by years of abusive drinking and accumulated lifetime exposure to alcohol.

Statistically significant relationships were observed between neuropsychological test scores and post-treatment alcohol consumption determined eight months after completing treatment for 72 alcoholics. These results, however, were influenced by the particular measure of post-treatment consumption evaluated, type of statistical analysis, and whether the entire sample of 72 or a sub-sample of 55 with consistently reported drinking information was used. Scores of tests most consistently showing relationships were often counter to the notion that increased neuropsychological performance predicts more favorable treatment outcome. Discriminant analysis resulted in 70 percent correct classification, with chance being 50 percent. It is concluded that neuropsychological evaluation is of limited clinical utility in predicting post-treatment alcohol consumption.

Significance to Biomedical Research and the Program of the Institute:

Recent and chronic drinking practices appear to have adverse effects on brain function in male alcoholics. Insofar as decisions about the initiation of therapeutic interventions which rely on cognitive processes are based on neuropsychological performance, we conclude that treatments may commence as soon as the clinical symptoms associated with acute withdrawal have subsided. Continued alcohol consumption by recovering alcoholics might serve to maintain cognitive performance at reduced levels, and this possibility should be considered in determining appropriate treatment goals for alcoholic patients. Neuropsychological evaluation is of limited clinical utility in predicting post-treatment alcohol consumption. However, it may be of value in assisting treatment staff in obtaining cognitively appropriate post-treatment employment for patients and in more effectively individualizing treatment, but this remains to be demonstrated.

Proposed Course:

Data analysis will be continued, and the results will be published in appropriate scientific journals.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00250-08 LCS

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Electrophysiological Studies of Acute and Chronic Alcohol Consumption

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	M. Eckardt	Section Chief	LCS, NIAAA
Others:	M. Linnoila	Scientific Director	NIAAA
	S. Adams	IRTA Fellow	LCS, NIAAA
	D. Rio	Research Physicist	LCS, NIAAA

COOPERATING UNITS *(if any)* Department of Psychiatry, Washington University (J. Rohrbaugh); Department of Psychology, Catholic University (R. Parasuraman); Department of Electrical Engineering, University of Nebraska (J. Varner); Neuroscience Research Branch, NIDA (J. Stapleton)

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section on Clinical Brain Research

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL MAN-YEARS: 3.2	PROFESSIONAL: 1.2	OTHER: 2.0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

- ☒ (a1) Minors
- ☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The aim of this research is to study the covert brain processes that underlie cognition and performance in human subjects, and the acute and chronic effects of ethanol upon such processes. Included is an extensive study in which we are examining brain processes in individuals with different family histories of alcoholism. A principal focus of these studies is the measurement of brain electrical potentials, which provide information regarding the timing and character of the constituent sensory, cognitive and motor elements that are the mechanisms underlying observable behavior. The study of the brain potentials also allows inference of the specific brain regions affected by ethanol. The brain electrical potentials are studied within a broad context provided by performance and psychometric data, and measurement within other psychophysiological response systems.

We have obtained data which document a large number of acute and chronic effects of ethanol on specific brain functions, ranging from sensory input to motor control functions. Of particular interest is a finding that brain electrical and autonomic signs of alerting and orienting are enhanced by ethanol, in contrast to its depressant effect on most other functions. A similar effect was observed in a sample of chronic alcoholic, organic brain disorder patients. Such findings suggest that ethanol intoxication and alcoholic organic brain disease may be associated with a disinhibition or deregulation of orienting processes.

Project Description:

Investigators:

M. Eckardt	Section Chief	LCS, NIAAA
S. Adams	IRTA Fellow	LCS, NIAAA
M. Linnoila	Scientific Director	NIAAA
R. Parasuraman	Professor	Catholic Univ., Washington, DC
D. Rio	Research Physicist	LCS, NIAAA
J. Rohrbaugh	Associate Professor	Washington Univ., St. Louis, MO
J. Stapleton	Staff Fellow	NRB, NIDA
J. Varner	Associate Professor	Univ. of Nebraska Omaha, NE

Objectives:

This research aims to provide a comprehensive overview of the effects of alcohol, both acute and chronic, on sensory, cognitive and motor systems. A primary focus is on event-related brain electrical potentials (ERPs), elicited in response to environmental stimulation, and extracted by computer from the on-going EEG. The electrical responses are studies in various tasks and under various conditions for information about the contributory neural processes and possible disturbances associated with alcohol consumption. Measurement of the brain electrical potentials is accompanied by simultaneous measurements of psychophysical judgments, autonomic and somatic systems.

Methods Employed:

Brain electrical activity and other psychophysiological responses are measured from surface electrodes using conventional EEG and polygraph instruments, allowing data to be acquired simultaneously from as many as 40 channels. The responses are analyzed with respect to wave form and sensitivity to experimental variables using multivariate techniques. Topographic distributions of the responses over the scalp are studied for evidence of neural sources of the electrical activity, using scalp mapping and dipole inference techniques.

Sensory functions are evaluated separately for visual, acoustic and somatosensory systems using clinically validated techniques. Visual stimulation and recording techniques permit evaluation of function in retina, optic nerve and tract, and cortical centers. Similarly, auditory and somatosensory techniques permit examination of function in peripheral, brainstem and cortical areas. Cognitive function is assessed by examining responses that are related to attention and judgment using paradigms in which information content and relevance are manipulated.

Noteworthy progress has been made on two methodological problems. One is the development of methods for objective classification of EEG phenotypes, using a parametric method of estimating frequency composition based on autoregressive filtering. This method is currently being expanded to allow for assessment of both temporal and spatial EEG nonstationarities, for possible genetic analysis. A second area of notable progress is in the installation and application of computer software for analysis of electromotive sources of brain electrical signals. This software is being elaborated to provide objective criteria for estimation of the number of contributory sources.

Major Findings:

1) Genetics of human EEG and ERPs. This study is conducted with Dr. M. Enoch and Dr. D. Goldman from the Section of Genetic Studies, LCS, and has entailed the acquisition of EEG and ERP signals under various conditions from a preliminary sample of more than 100 individuals drawn from more than 40 different families. In accord with previous findings, a number of distinctive EEG phenotypes have been identified and distributions of these phenotypes in other family members are being studied. A number of families have been identified in which specific phenotypes appear to be segregating. In addition, we are proceeding with the development of alternatives schematas for the identification and classification of EEG and EEG phenotypes, including the objective method described above.

2) ERP signs of orienting in alcoholic chronic brain disorder (OBS). In a study of the 0 wave to infrequent visual and auditory stimuli, it was found that the 0 wave was markedly enhanced in groups of patients diagnosed with Korsakoff's syndrome and alcoholic dementia, in comparison to a group of age-matched normals. A simple measure based on 0 wave amplitude correctly classified more than 90% of the normal and patient subjects. The enlarged 0 wave in the patients was accompanied by exaggerated heart rate responses, particularly the acceleration component peaking at about 2 seconds post stimulus. The 0 wave enlargement is similar, although of less extent, to that we have previously observed in normal subjects during light sleep. These data are interpreted in terms of inadequate control over the orienting response, perhaps reflecting a frontal lobe deficit. Consistent with this hypothesis, the 0 wave amplitude is highly correlated with performance scores on standardized neuropsychological tests of frontal lobe function. In addition, it has been determined that 0 wave amplitude is highly correlated with memory performance. The relationship with memory performance appears to be specific to the alcoholic OBS patients, since no 0 wave enhancement has been obtained in a recently-begun study of Alzheimer's dementia patients.

3) Relationship of EEG to Positron Emission Tomography (PET) measures in alcoholic chronic OBS. A PET study of alcoholic OBS patients have revealed a number of subtle differences between patients and age-matched normal subjects. We have recently completed an analysis of the EEG data obtained simultaneously during the 40 minute isotope uptake period. Whereas spectral estimates of EEG frequency and amplitude are not greatly different if averaged over the entire 40 minute period, analysis of shorter epochs within the period reveals highly significant differences between normals and OBS patients. The normal subjects show a pronounced depression of EEG amplitude during a ten minute epoch immediately following injection. This transient depression is not apparent in the records from OBS patients. These EEG differences suggest the

presence of differences in global response to the PET injection procedures, which may contribute to the observed PET differences.

4) ERPs in normal and in Attention Deficit/Hyperactivity Disorder children. An experimental protocol has been developed to provide a battery of behavioral and electrophysiological measures of attentional performance in children. An initial finding with a group of normal children indicates that the 0 wave in children is substantially larger than in adults.

Publications:

Eckardt MJ, Rohrbaugh JW, Rio DE, Martin PR. Positron emission tomography as a technique for studying the chronic effects of alcohol on the brain, Ann Med 1990;22:341-6.

Johnson R Jr, Rohrbaugh JW, Ross JL. Event-related potential activity in patients with Turner's syndrome. In: Brunia CHM, Gaillard AWK, Kok A, eds. Psychophysiological Brain Research, Tilberg: Tilberg University Press, 1990;180-3.

Rohrbaugh JW, Parasuraman R, Johnson R Jr, eds. Event-related brain potentials: Basic issues and applications. New York: Oxford University Press, 1990.

Rohrbaugh JW, Varner JL, Paige S, Eckardt MJ, Ellingson RJ. Event-related perturbations in an electrophysiological measure of auditory sensitivity: Effects of probability, intensity, and repeated sessions, Int J Psychophysiol 1990;10:17-32.

Rohrbaugh JW, Varner JL, Paige SR, Eckardt MJ, Ellingson RJ. Auditory and visual event-related perturbations in the 40 Hz auditory steady state response, Electroencephal Clin Neurophysiol 1990;76:148-64.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00267-06 LCS

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Brain Imaging

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	M. Eckardt	Section Chief	LCS, NIAAA
Others:	M. Linnoila	Scientific Director	NIAAA
	R. Rawlings	Mathematical Statistician	LCS, NIAAA
	D. Rio	Physicist	LCS, NIAAA

COOPERATING UNITS *(if any)*

Diagnostic Systems Branch, NIDR (U. Ruttimann)

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section on Clinical Brain Research

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	1.0	3.0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Various clinical imaging methods are being used to study the brain _in vivo_. These techniques enable comparisons of gross anatomy (CAT-Computed Axial Tomography; MRI - Magnetic Resonance Imaging) of the brain with electrical activity (EEG - electroencephalography; ERPs-Event-Related Potentials) and rate of glucose utilization in a specific regions (PET -Positron Emission Tomography). From a clinical perspective, these techniques, in association with other diagnostic tests, enable qualitative judgments to be made as to the anatomic and physiologic integrity of the brain. In order to quantitatively analyze image data, the imaging techniques themselves are being investigated, as well as the effects of the associated mathematical models and subjective inputs on the reconstruction of the brain image. Moreover, mathematical and statistical methods for evaluating and relating these various sources of multivariate data are being developed.

Project Description

Investigators:

M. Eckardt	Section Chief	LCS, NIAAA
M. Linnoila	Scientific Director	NIAAA
R. Rawlings	Mathematical Statistician	LCS, NIAAA
D. Rio	Physicist	LCS, NIAAA
U. Ruttimann	Biomedical Engineer	DSB, NIDR

Objectives:

Our goals are 1) to develop three-dimensional PET and CAT/MRI images which are superimposable, and 2) to develop new mathematical and statistical methods to assess these different types of multivariate image data and determine relationships between these images and brain electrical activity monitored at the scalp.

Methods Employed:

A critical review of the raw and reconstructed data obtained from PET, CAT, and MRI is being carried out to insure that sources of noise inherent in each technique are taken into account, thereby insuring that artifacts will be correctly eliminated and confidence intervals may be more accurately estimated, leaving only statistically significant differences. Emphasis is currently on self-attenuation within the brain-skull system, applicability of the glucose utilization model, and software filters used to reconstruct images.

Two of the main issues involved in this area are patient position monitoring during a scan and patient repositioning, either on the same scanner at different times or on another scanner. Currently, two methods of recording patient position are being tested. The first system consists of a magnetic transducer attached to the patient, with associated pickup attached to the various scanners which will enable us to determine the position of a "stable point" on the patient, and a computer system which will display this information both numerically and graphically. The second system consists of a mechanical system designed to ride on the patient's face to provide more quantitative visual cues as to patient movement without the complications of the aforementioned system.

All data obtained from the scanners are processed and displayed on our own image processing system enabling us to analyze basic pixel data instead of post-processed images. Individual brain slices are oriented in three dimensions and displayed as stacked three-dimensional data or as surface contours. Using these methods, it will be possible to display and calculate regional volumes and superimpose data from CAT, PET, and MRI, as well as other sources. This will insure that a regional alteration in metabolism corresponds to a particular anatomic location.

Current research efforts in this area include a review of (1) three-dimensional registration methods which purportedly match associated brain image volumes acquired from various imaging modalities, and (2) associated assumptions in the models used to reorient and resample data originally taken in one orientation at a given resolution. In particular, the registration techniques being developed use surface normals of external and internal brain anatomy or pseudoanatomy, and this technique is being compared to other, less elaborate methods already developed. These algorithms can also be used to overlay brain images from the same patient taken at different times. Measurement criteria for goodness of registration are also being developed. For SPECT, because of its lower spatial resolution, these techniques are being modified to use external control structures placed on the patient when they are scanned to provide registration landmarks.

Two other major areas of research associated with image analysis are being investigated. The first is image segmentation, which consists of semiautomated identification of major areas (white and grey matter, CSF) or landmarks of brain anatomic or metabolic topology. The second is tangential to the problem of volume image registration across modalities within a subject and addresses the concern of consistent data extraction across subject groups. This problem has been partially addressed by the previously mentioned rescaling procedure, however, insofar as metabolic images are confounded by anatomic information, a solution to this problem requires the use of multimodality data. Current and future research to solve this problems will consist of mapping volumetric CT or MRI images to a common anatomic atlas using spatial correlation and local warping transformations.

Resulting data are analyzed with mathematical techniques used in image processing, pattern recognition, and spectral analysis, i.e., by representing the spatial data in frequency space. Rigorous statistical tests are then used to study differences between various clinical populations.

Procedures are being developed to calculate and display scalp-monitored electrical potential fields and to estimate the position and distribution of electrical sources in the brain producing these potentials. This will enable us to correlate anatomic or metabolic changes in the brain with modifications of cognitive processes as represented by changes in evoked responses.

Major Findings:

Data for this study are being collected and analyzed. We have developed methods to analyze image data acquired from groups of carefully defined subjects. This had involved (1) the use of two-dimensional registration and rescaling algorithms, which enable group averaging and increase the signal-to-noise ratio of the data, and (2) the application of frequency analysis to study the basic underlying informational patterns embedded in the brain images. Rescaling, as discussed here, consists of applying translation, rotation and magnification transformations in two dimensions until comparable brain image slices fill the same region in space. These techniques also enable more rigorous statistical procedures to be applied and has led to techniques which enhance the quality of the averaged group images, verify basic resolution parameters of the scanner systems, and provide a statistical test for discrimination between groups.

Significance to Biomedical Research and the Program of the Institute:

Establishing structure and function relationships among various areas of the brain is a crucial step in determining mechanisms. The approach advocated in the research described herein emphasizes 1) detailed and intensive assessment of relatively few, carefully selected patients, thereby reducing heterogeneity in patient characteristics and enabling a convergence of information, and 2) comparing three-dimensional PET, CAT, and MRI images with each other and with electrical sources derived from scalp-monitored EEG and ERPs. Such studies have yet to be reported in the literature. Successfully combining these techniques would be a significant accomplishment with obvious applicability to other studies of brain structure and function.

Proposed Course:

Data are being collected and analyzed. As analyses are completed, the results will be published in appropriate scientific journals.

Publications:

Eckardt MJ, Rohrbaugh JW, Rio DE, Martin PR. Positron emission tomography as a technique for studying the chronic effects of alcohol on the brain, Ann Med 1990;22:341-6.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00268-06 LCS

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Behavioral Effects of Ethanol and Other Psychotropic Drugs

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	M. Eckardt	Section Chief	LCS, NIAAA
Others:	M. Durcan	Visiting Associate	LCS, NIAAA
	D. Goldman	Section Chief	LCS, NIAAA
	M. Linnoila	Scientific Director	NIAAA

COOPERATING UNITS *(if any)*

VA Medical Center, Portland, OR (J. Crabbe);
Laboratory of Clinical Physiology, NIA (H. Weingartner)

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section on Clinical Brain Research

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.4	1.4	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Pharmacologic and genetic methods are being used to determine the psychobiological mechanisms underlying various behavioral processes. The current research is focused on the neurobiology of anxiety, impulsivity and aggression in mice and on learning and memory processes in humans. The effects of ethanol, and of drugs with known and specific mechanisms of action are being investigated.

Selective alpha-2 adrenoceptor antagonists have been shown to reduce ethanol's hypothermic and ataxic effects but not to alter ethanol's anxiolytic, anticonvulsant or locomotor stimulant effects. The effect is mediated centrally rather than peripherally and appears to involve alpha-2 adrenoceptors rather than imidazoline binding sites.

Male mice that show high levels of aggression towards their cage-mates differ from both their subordinate cage-mates and from control mice housed in cages in which little fighting is observed. The most consistent difference is observed in Porsolt's swim test where subordinate mice show increased immobility relative to controls. The behavioral differences appear to follow rather than precede the aggressive social interactions.

Project Description:

Investigators:

M. Eckardt	Section Chief	LCS, NIAAA
J. Crabbe	Staff Scientist	VA Med. Ctr., Portland, OR
M. Durcan	Visiting Associate	LCS, NIAAA
D. Goldman	Section Chief	LCS, NIAAA
M. Linnoila	Scientific Director	NIAAA
H. Weingartner	Section Chief	LPC, NIA

Objectives:

The behavioral effects of ethanol in rodents have been most extensively studied using tests sensitive to the drug's hypnotic, hypothermic, psychomotor impairing, and sedative effects. In contrast, ethanol's anxiolytic, anticonvulsant, amnestic and reinforcing effects have been investigated less thoroughly, largely because tests for these effects are more difficult to develop. We are developing tests suitable for investigating these latter effects in mice. Following the validation of specific tests, we will investigate the relationship between ethanol's various behavioral effects using pharmacological and behavioral genetic methods.

It has been suggested that alcoholics can be divided into at least two different groups. One group is characterized as having a late onset of ethanol-related problems and show high levels of harm avoidance. These alcoholics may drink for ethanol's anxiolytic effects. The other group is characterized by impulsive and often aggressive behavior. There may be a neurochemical bases for these two forms of alcoholism. We have been developing animal models of anxiety, aggression and impulsive behavior to better study these two different forms of alcoholic behaviors. In each model, we examine the effects of ethanol and compare the behavior of different populations of mice. Both environmental and genetic manipulations are used to alter mouse behavior. Behavioral genetic techniques are then applied in an attempt to understand the mechanisms underlying individual differences.

Several lines of evidence suggest that there are a number of different forms of learning and memory. For example, a person may appear to be amnesic when a test of episodic learning is used, but perform normally in a test of procedural learning. In the present series of studies, we are attempting to determine which cognitive processes can function independently. We believe that cognitive dysfunction can yield information about normal cognitive functioning. Therefore, we are comparing the performance of several populations of subjects in a battery of cognitive tasks.

Subjects include normal volunteers under the influence of either ethanol, benzodiazepines or an anticholinergic drug (scopolamine), and patients with various memory disorders including Korsakoff's disease, Alzheimer's disease and alcoholic dementia. By determining what these populations can and cannot learn and remember, we expect to provide evidence that some cognitive processes function independently of others and uncover the neurobiological mechanisms mediating these different processes.

Methods Employed:

The behavioral tests currently being used in mice are:

1) The plus-maze test of anxiety is used to assess anxiolytic effects of various drugs. Mice are placed on an elevated plus-maze consisting of two open and two enclosed arms. The proportion of time spent on the open arms expressed as a percentage of the total time on both types of arms issued as an index of anxiety.

2) A holeboard test in which mice are placed in a plexiglass box containing four holes and their directed exploration (head-dipping) is assessed independently of locomotor activity.

3) A test of social and aggressive behavior that involves placing pairs of male mice in an unfamiliar arena, and scoring their behavior for a period of 5 min. using a keyboard.

4) An observer-rated test of ataxia and a test of the duration of loss-of-righting reflex observed following administration of drug.

5) A seizure threshold paradigm in which one of a number of different convulsants is infused through the tail vein of mice and the latency to seize is measured.

6) Porsolt's swim test in which a mouse is placed in a pail of water and the duration of immobility is measured.

The experimental manipulations involve pharmacological treatments with various drugs with known mechanisms of action, environmental manipulations such as isolation, and genetic manipulations that include the use of different inbred strains of mice.

An extensive test battery for assessing learning and memory in humans has been developed. The tests used assess aspects of episodic memory, procedural learning, several different forms of priming, retrieval of information from semantic memory and recognition memory.

Major Findings:

A subpopulation of aggressive NIH Swiss mice(referred to as alpha-mice) differ in behavior and neurochemistry from both non-aggressive control mice and from the mice they chronically defeat. The alpha-mice spent less time immobile in the swim test, and spent less time head-dipping in the hole board than either controls or submissive mice who spent more time immobile in the swim test than controls and had elevated concentrations of the serotonin metabolite 5-HIAA in hypothalamus, hippocampus and brain stem structures. There were no differences between the groups in serotonin or noradrenaline concentrations in mice before group housing indicating that the behavioral differences between the alpha-and subordinate mice followed rather than preceded the social interactions.

Alpha-2 adrenoceptor antagonists atipamezole and idazoxan reduced the ataxic and hypothermic effects of ethanol, but failed to alter ethanol's anxiolytic action in the plus-maze test or anticonvulsant action against seizures caused by bicuculline. The effects appear to be mediated centrally rather than peripherally since L 659,066, an alpha-2 adrenoceptor antagonist that does not cross the blood/brain barrier,failed to reduce ethanol's effects. Ethanol's antagonizing action appears to be mediated by alpha-2 adrenoceptors rather than imidazoline binding sites since RX 821002, an alpha-2 adrenoceptor antagonist with low affinity for imidazoline binding sites, was also capable of reducing ethanol's ataxic and hypothermic effects. The results indicate that central adrenergic mechanisms play a role in some, but not all of the acute effects of ethanol.

Significance to Biomedical Research and the Program of the Institute:

Cloninger has proposed that alcoholics can be divided into two types. Type 1 alcoholics have been classified as anxious and often drink to relieve anxiety. Type 2 alcoholics are impulsive and are often aggressive. We are developing and using animal models of anxiety and impulsive/aggressive behavior in an attempt to understand the genetic and environmental variables that may be important in the different forms of alcoholism.

The neurobiological mechanisms underlying ethanol's many behavioral effects are poorly understood. We believe we may gain insight into these mechanisms by examining the interactions of ethanol with drugs with highly specific and well-characterized mechanisms of action. Our understanding of these mechanisms will be invaluable in the development of drugs that might reduce voluntary ethanol consumption, and drugs that might antagonize the potentially lethal effects of high doses of ethanol.

The ability to distinguish between various basic processes involved in learning and memory is of fundamental importance in the diagnosis and treatment of memory disorders which often arise as a complication of chronic alcoholism. We hope to identify and investigate the neurobiology of distinct cognitive processes using drug studies in normal volunteers. This will allow a more efficient screening of potential treatments.

Publications:

Hilakivi LA, Lister RG, Durcan MJ, Ota M, Eskay RL, Mefford I, Linnoila M. Behavioral, hormonal and neurochemical characteristics of aggressive alpha mice, Brain Res 1989;502:158-66.

Hilakivi LA, Lister RG. Comparison between BALB/cJ and BALB/cBy mice in tests of social behavior and resident-intruder aggression, Aggressive Behav 1989;15:273-80.

Hilakivi LA, Lister RG. The effect of ethanol on the social behavior of group-housed and isolated mice, Alcoholism: Clin Exp Res 1989;13:622-5.

Hilakivi LA, Durcan MJ, Lister RG. Effects of ethanol on fight- or swim-stressed mice in Porsolt's swim test, Neuropsychopharmacol 1989;2:293-8.

Lister RG. Antagonizing the behavioral effects of drugs: a discussion with specific reference to benzodiazepines and alcohol, J Psychopharmacol, 1989;3:21-8.

Mefford I, Lister RG, Ota M, Linnoila M. Antagonism of ethanol intoxication in rats by inhibitors of phenylethanolamine N-methyl transferase, Alcoholism: Clin. Exp. Res. 1990;14:53-7.

Durcan MJ, Lister RG, Linnoila M. Behavioral effects of the inhibitors of phenylethanolamine N-methyl transferase, LY 78335 and LY 134046, and their interactions with ethanol, Psychopharmacology, 1990;101:196-202.

Hilakivi LA, Lister RG. Correlations between behavior of mice in Porsolt's swim test and in tests of anxiety, exploration and locomotion, Behav Neural Biol 1990;53:153-9.

Linnoila M, Stapleton, JM, Lister RG, Moss H, Lane E, Granger A, Greenblatt DJ, Eckardt MJ. Effects of adinazolam and diazepam, alone and in combination with ethanol, on psychomotor performance and on autonomic nervous system reactivity in healthy volunteers, Eur J Clin Pharmacol 1990;38:371-7.

Lister RG. Ethologically-based animal models of anxiety disorders, Pharmacol Ther, 1990;46:321-40.

Lister RG. Alcohol and anxiety, Clin Neuropharm 1990;Suppl 2, 504-5.

Lister RG, Gorenstein C, Risher-Flowers D, Weingartner HJ, Eckardt MJ. Dissociation of the acute effects of alcohol on implicit and explicit memory processes, Neuropsychologia, in press.

Hilakivi-Clarke LA, Turkka J, Lister RG, Linnoila M. Effects of early postnatal handling on brain beta-adrenoceptors and behavior in tests related to stress, Brain Res, in press.

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Lister RG. The effects of benzodiazepines and 5-HT1a agonists on learning and memory. In: Rodgers RJ, Cooper SJ, eds. 5-HT1a agonists, 5-HT3 antagonists and benzodiazepines, Wiley, in press.

Lister RG, Linnoila M. Alcohol, the chloride ionophore, and endogenous ligands for benzodiazepine receptors. Neuropharmacology, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00275-03 LCS

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Psychomotor and Cognitive Aspects of Alcoholism

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	M. Eckardt	Section Chief	LCS, NIAAA
Others:	D. George	Acting Section Chief	LCS, NIAAA
	M. Linnoila	Scientific Director	NIAAA

COOPERATING UNITS *(if any)*

Georgetown University, Washington, DC (S. Gilson); VAMC, Charleston, SC (B. Adinoff); Washington University, St. Louis, MO (J. Rohrbaugh)

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section on Clinical Brain Research

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL MAN-YEARS: 2.8	PROFESSIONAL: 2.2	OTHER: .6

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This series of studies is designed to investigate the neuroanatomical and neurochemical pathways underlying impaired cognitive and psychomotor functions in detoxified adult alcoholics and their offspring. We have demonstrated that long-term alcohol abuse is associated with unusual saccadic eye movements in about half the alcoholics studied. Low doses of diazepam administered i.v. to these alcoholics reduce the number of unusual eye movements. It is anticipated that studies on the children of alcoholics will clarify whether these unusual eye movements predate the onset of excessive alcohol consumption.

Project Description

Investigators:

M. Eckardt	Section Chief	LCS, NIAAA
B. Adinoff	Chief	VAMC, Charleston, SC
D. George	Acting Section Chief	LCS, NIAAA
S. Gilson	Assistant Professor	Georgetown U., Washington, DC
M. Linnoila	Scientific Director	NIAAA
J. Rohrbaugh	Associate Professor	Washington Univ., St. Louis, MO

Objectives:

The present series of studies is designed to examine the neurochemical and neuroanatomical changes which underlie the cognitive and psychomotor deficits in detoxified adult alcoholics with and without severe cognitive dysfunction, and in children at risk for alcoholism.

Methods Employed:

Long-term, excessive alcohol consumption has been associated with well-documented adverse effects upon brain functions. We are interested in specifying the types of functions which become compromised so that we can precisely define the anatomical pathways and neurochemical systems involved. To accomplish this goal, we have developed and continue to develop a series of standardized cognitive and psychomotor tasks. Performance on each task allows us to infer which neuroanatomical or neurochemical pathway may be functioning irregularly. For example, research on nonhuman primates indicates that each of three of the memory tasks in our battery, delayed matching-to-sample, spatial delayed response, and twenty-four hour concurrent discrimination, relies on a distinct neuroanatomical pathway; the performance of a particular patient population on these tasks will therefore allow us to infer how well these pathways may be functioning. Furthermore, we intend to combine our cognitive and psychomotor tasks with the administration of drugs which are known to affect specific neurotransmitter systems. Combining drugs with our tasks in normal volunteers may show us the role of specific transmitter systems in these tasks; combining drugs with our tasks in patient populations may provide a basis for remedial or preventive therapy.

Currently, particular attention has been focused on memory function and saccadic eye movements. A standardized battery of memory tests based primarily on recent research with nonhuman primates has been developed. This research has shown that there are several types of memory and that each type of memory is served by unique neuroanatomical and neurochemical pathways. We are particularly interested in determining whether specific memory systems, and therefore neuroanatomical and neurochemical pathways, may be compromised in detoxified alcoholics. This work is still in progress. In addition to the memory tasks, standard psychomotor tasks have been used to probe motor pathways. A new method of analyzing saccadic eye movements, based upon their velocity profiles, has been developed.

Major Findings:

We have completed analysis of the effects of diazepam on the saccadic eye movements of detoxified alcoholic adults and controls. It is well known that diazepam decreased the peak velocity of saccadic eye movements. Our results show that this slowing is due to a change in the type of eye movements rather than a slowing per se. Saccades are produced by one or more pulses of force delivered to the extraocular muscles (this can be ascertained by examining the velocity profile of the saccade). Most saccades, especially those less than 10 degrees in excursion, are produced by a single force pulse. Whenever a single pulse is used, there is a simple linear relationship between the peak velocity and the amplitude of the movement. Multiple pulses are necessary whenever a) feedback is used to control the movement of the eye, or b) the extent of the saccade is so large that the force necessary to move the eye in a single pulse exceeds the maximum which can be delivered by the extraocular muscle system. In the second case, the system delivers two or more pulses rather than prolonging the first pulse. Although single pulse saccades tend to predominate at shorter amplitudes and multiple pulse saccades at longer amplitudes, their ranges overlap extensively. At equal amplitudes, single pulse saccades have higher peak velocities than multiple pulse saccades.

Diazepam administered i.v. in control subjects decreases the percentage of saccades which are of the single pulse type and also decreases their range of amplitude; the opposite is true of multiple pulse saccades. As a result, peak velocity is slowed, on average. The relationship between percentage of single pulse saccades and dose of diazepam appears to be approximately linear. There is no slowing of the peak velocity of single pulse saccades following diazepam administration. These results are consistent with the notion that diazepam decreases the peak force which can be delivered by the extraocular muscle system. Since the latency for making single pulse saccades is not different from that for making multiple pulse saccades, it is unlikely that multiple pulse saccades are preprogrammed.

About half of the detoxified alcoholics tested responded like the controls. The other half showed a U-shaped response to the drug. In the absence of diazepam, these subjects made more multiple pulse saccades; at low doses, they appeared more normal (more single pulse saccades); and at higher doses they again made more multiple pulse saccades. Additional subjects are needed to determine what other factor or factors may discriminate these two groups of alcoholics.

Significance to Biomedical Research and the Program of the Institute:

Long-term alcohol abuse has adverse effects upon cognitive and psychomotor functions. Fifty to seventy-five percent of alcoholics have impaired cognitive function, and ten percent have alcoholism-associated organic brain syndromes. Pairing cognitive and psychomotor tasks with drugs which affect specific transmitter systems may indicate both the role of specific transmitters in these tasks as well as the neurochemical deficits present. If this can be accomplished, we will be in a better position to provide both preventive and remedial treatment.

Some of the deficits present in detoxified alcoholic adults appear to be present in the offspring of alcoholics prior to any alcohol consumption. It is important to distinguish which deficits are present prior to abuse and which arise afterwards. For those deficits present prior to abuse, it may be of value to distinguish those which may be associated with subsequent abuse and those which are merely correlated. For these reasons, we are planning to begin studies of the children of alcoholics.

Proposed Course:

Data will continue to be collected, and the results will be published in appropriate scientific journals.

Publications:
None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00237-09 LCS

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Individual Variability in Drug Metabolism by Carbon Dioxide Breath Test

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	S. Shoaf	Acting Chief	LCS, NIAAA
Others:	T. Hart	Chemist	LCS, NIAAA

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section of Clinical Biochemistry and Pharmacology

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL MAN-YEARS: 1.8	PROFESSIONAL: 0.8	OTHER: 1.0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project has been terminated because of the lack of suitable test substrates. Literature searches revealed that the rate of metabolism of caffeine is significantly affected by caffeine ingestion, smoking and other substances that induce P450IA2 isozymes (e.g. char broiled beef). The variation in rates of caffeine metabolism in non-smoking volunteers encompasses the range of metabolism rates determined in smokers. Caffeine is not a good substrate for the determination of baseline rates of liver function as its rate of metabolism is too variable. A systematic search of the literature for compounds that may be demethylated by the P450IIE isozymes (i.e. induced by ethanol) revealed none suitable for human use; considered test compounds were found to be either carcinogenic or toxic.

This project has been terminated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00292-01 LCS

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Mechanisms Responsible for Altered Drug Metab. Following Withdrawal from Ethanol

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	S. Shoaf	Acting Section Chief	LCS, NIAAA
Other:	C. Niebylski	NRC Fellow	LCS, NIAAA
	B. Song	Section Chief	LMMB, NIAAA

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Clinical Studies

SECTION
Section of Clinical Biochemistry and Pharmacology

INSTITUTE AND LOCATION
NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	2.0	0.5

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A protocol was submitted to the Animal Care and Use Committee for approval. A literature search has been completed. Substrates specific for cytochrome P450 isozymes have been identified. Some of the in vitro enzyme assays have been developed and validated. It is expected that animal exposure studies will begin soon.

Project Description:

Investigators:

S. Shoaf	Acting Section Chief	LCS, NIAAA
C. Niebylski	NRC Fellow	LCS, NIAAA
B. Song	Research Biochemist	LMMB, NIAAA

Objectives:

To determine the mechanisms responsible for the altered metabolism of therapeutic agents following ethanol withdrawal. Animal studies will be used to guide the development of human research protocols.

Initial studies will measure cytochrome P450 levels and activities, in vitro, and membrane lipid composition and fluidity in the rat following chronic ethanol exposure and at various times following the withdrawal of ethanol. Although the physiological changes that occur during the development of dependence are well characterized, to our knowledge, there are no studies that have examined P450 activity or changes in membrane lipid composition during and after acute withdrawal.

Methods Employed:

Rats will be administered ethanol as part of a liquid diet; the Lieber-DeCarli diet, 36% of calories as ethanol, will be given for 21 days. Exposed and control animals will be pair-fed with the control diet isocalorically supplemented with dextrin-maltose. An inhalation exposure model will also be tested for its ability to induce metabolic changes similar to the liquid diet. Inhalation exposure would avoid the necessity of pair feeding and allow us to explore how different diets alter the effect of ethanol. Microsomal preparations will be made from rat tissues. Rats will be killed immediately after the last feed period and at various times following ethanol withdrawal.

The activity of various cytochrome P450 isozymes will be measured in vitro using microsomal enzyme assays. By following the formation rate of a metabolite that is formed via the action of a single cytochrome P450 isozyme, the activity of that isozyme can be characterized. For example, acetanilide hydroxylation is mediated by the P450IA2 isozyme. The demethylation of nitrosodimethylamine at low concentrations is mediated by P450IIE2 (the cytochrome P450 induced by ethanol). P450IIE also largely mediates the hydroxylation of p-nitrophenol (>90%).

Antibodies will be used to immunoquantitate the amount of specific P450 isozymes. Western blots with computerized densitometry as well as immunoprecipitation and radioimmunoassay may be utilized.

Changes in membrane composition will be determined by extracting lipids and analyzing the fatty acid distribution using gas chromatography with flame-ionization detection.

The fluidity of microsomal preparations will be determined by incubating microsomes with lipophilic, fluorescent probes such as 1,6-diphenyl-1,3,5-hexatriene (DPH). The fluorescence depolarization of the probe will be measured using both steady state and time resolved fluorescence anisotropy with a Photon Technologies International (Princeton) LS-100 Spectrofluorimeter.

Major Findings:

This project has been recently initiated. A review of the literature has been completed and suggests that cytochrome P450 levels and activities in abstinent alcoholics may not rapidly return to control values. There are no reports of studies that have followed cytochrome P450 levels or activities following ethanol withdrawal. It will be possible to survey the activities of specific cytochrome P450 isozymes without having to isolate each specific form. By following the formation rate of a metabolite that is formed via the action of a single cytochrome P450 isozyme, the activity of that isozyme can be characterized.

Significance to Biomedical Research and the Program of the Institute:

It has been reported that the efficacy of some psychotropic agents is lower in abstinent alcoholics compared to controls. It has been proposed that therapeutic concentrations are not obtained in abstinent alcoholics when they are dosed at levels normally given controls. The mechanism responsible for the lowered steady state concentrations is proposed to be an increased rate of metabolism. It is important to understand the physical mechanisms underlying altered drug metabolism so that appropriate adjustments can be made in the dosage regimens of therapeutic compounds used for the treatment of abstinent alcoholics.

Proposed course:

Additional *in vitro* enzyme assays will be developed and validated. Rats will be exposed and withdrawn from alcohol, tissues will be collected and prepared for analysis. Evaluation of the changes that occur following the inhalation of ethanol will be compared to those that occur following consumption of ethanol in a liquid diet.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 AA 00285-02 LCS

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Physiological Functions of Lipoxygenase Products

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. Karanian	Research Physiologist	LCS, NIAAA
Others:	H-Y Kim	Research Chemist	LCS, NIAAA
	N. Salem, Jr.	Section Chief	LCS, NIAAA

COOPERATING UNITS *(if any)*
Laboratory of Immunology, NIDR (L. Wahl);
Receptor Labs (P. Swann)

LAB/BRANCH
Laboratory of Clinical Studies

SECTION
Section of Analytical Chemistry

INSTITUTE AND LOCATION
NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL MAN-YEARS: 1.2	PROFESSIONAL: 1.2	OTHER: 0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Mammalian platelets are high in 12-lipoxygenase activity and capable of enzymatically producing 13-OH-18:2n6 (13-HODE), 12-OH-20:4n6 (12-HETE), 12-OH-20:5n3 (12-HEPE), 11-OH-22:6n3 (11-HDHE) and 14-OH-22:6n3 (14-HDHE). In addition, we have reported the production of a 15-lipoxygenase product, 17-OH-22:6n3 (17-HDHE). The biological activities of these metabolites have been examined with emphasis on their possible mechanisms of action. HDHE's dose-dependently stimulate lipoxygenase products from lung parenchymal tissue, neutrophils and monocytes. A direct correlation exists between endogenous leukotriene production and the contractile properties of HDHE in airway smooth muscle. In addition, HDHE's may specifically interact with thromboxane receptors as suggested by their relatively specific inhibition of U46619-induced contractions in smooth muscle. Preliminary data demonstrate a direct relationship between inhibition of U46619-induced platelet aggregation and 3H-U46619 specific binding by relatively high concentrations of HDHE. Moreover, vasculature obtained from ethanol-dependent or fish oil-fed rats are less contractile to the thromboxane analog, U46619. Ionophore-stimulated 13-HODE and 12-HEPE production was depressed in platelets obtained from ethanol-dependent rats. The production of these hydroxy fatty acids from platelets was markedly greater when the platelet membrane was enriched with the appropriate n-6 or n-3 precursor fatty acid. Moreover, supplementation with corn oil (n-6) countered the ethanol- induced decline in platelet n-6 hydroxy fatty acid. Similarly, supplemention with fish oil (n-3) effectively countered the observed decline in the production of the n-3 hydroxy fatty acid indicating that increasing substrate availability through dietary means is an effective way of countering the effects of ethanol in this regard.

Project Description:

Investigators:

J. Karanian	Research Physiologist	LCS, NIAAA
H-Y Kim	Research Chemist	LCS, NIAAA
N. Salem, Jr.	Section Chief	LCS, NIAAA
P. Swann	Senior Scientist	Receptor Labs, Chicago, IL
L. Wahl	Section Chief	LMI, NIDR

Objectives:

In general, (i) to characterize the physiological functions of lipoxygenase products of n-3 and n-6 highly unsaturated fatty acids including eicosapentaenoic (20:5n3), docosahexaenoic (22:6n3) and arachidonic (20:4n6) acids. In particular, (ii) to study the modification by ethanol of the function of these metabolites in the nervous and cardiovascular systems as well as in other organs and cells, and (iii) to evaluate the potency of fatty acid supplementation with respect to countering fatty acid deficiencies caused by chronic ethanol exposure and the resulting alteration in eicosanoid/docosanoid mediated cellular responses.

Methods Employed:

Synthesis of Hydroxylated Docosahexaenoates

Washed platelets were prepared in a Tris-EDTA buffer and incubated for 30 min at 37°C with 200 mM 22:6n3. In some cases, rats were exposed to ethanol inhalation for 14 days in the presence of either a corn oil or fish oil diet for 28 days. These animals were placed in specially designed inhalation chambers using an ethanol vapor concentration sufficient to produce a blood alcohol level of >120 mg%. The effects of calcium ionophore A23187 on the yield of the hydroxylated products was studied by its addition in vitro. Reactions were terminated by acidification to pH 3.2 with formic acid. In some cases 500 ng of 5-HETE was added as an internal standard. The metabolites were then extracted twice with equal volumes of dichloromethane.

Analysis of Hydroxy Fatty Acids

The positional isomers of OH-22:6n3 (17, 14 and 11 HDHE) and other hydroxy fatty acids (13-HODE, 5,12, and 15-HETE, and 12-HEPE) were separated using an HP-1090 HPLC equipped with a diode array UV detector (235 nm). A 4.6 x 25 cm Axxichrom ODS column (5 mm) was used with a flow rate of 0.8 ml/min and a solvent system composed of 100 mM ammonium acetate and methanol. In some cases the hydroxy fatty acids of interest were collected and methylated using diazomethane in order to further purify and quantitate the HETE and HDHE. After dissolution in hexane, the stereoisomers of each of these products were separated on a dinitro- benzoylphenylglycine column (5 μ, 4.6 mm x 25 cm) using hexane-propanol-acetonitrile (497.5:5.2:0.5) as the mobile phase with a flow rate of 0.8 ml/min. In some cases radiolabeled 20:4n6 was used and the eluant was passed into a radioactivity detector.

Bioassays and Radioimmunoassays

Guinea pigs were used as a source of lung parenchymal strips and rats were sources of aortic rings. Animals were killed by decapitation, the preparations trimmed at 4°C and incubated in a Krebs-Ringer bicarbonate buffer. Changes in tension were monitored by use of force-displacement transducers. Human PMNs were obtained from whole blood using the Histopaque gradient method and human monocytes were obtained by elutriation. The effect of A23187, various precursor fatty acids and hydroxy fatty acids on leukotriene production were compared. In these cases the incubation medium was assayed for both peptidyl-leukotriene, leukotriene C_4 and leukotriene B_4 levels using relatively specific radioimmunoassay antibodies.

Dietary Studies

Rats were fed a purified diet similar in composition to the AIN-76A formulation, except that the fat content was raised to 10 wt%, the sucrose was reduced from 50 to 15 wt% and replaced with corn starch and a Teklad vitamin mix was used. Dietary fat was composed of 9 wt% of menhaden or corn oil in addition to the 1 wt% of corn oil in the basal mixture. Rats were fed these diets for 28 days after which they were sacrificed and their platelets analyzed for fatty acid content and A23187-stimulated lipoxygenase products. In some cases, groups of rats were placed in inhalation chambers the final 14 days of the dietary period either in the presence or absence of ethanol vapors.

Major findings:

As previously reported, the hydroxylated metabolites of 22:6n3, which are formed via a 12-lipoxygenase by human platelet, are biologically active. The contractile properties of 14-HDHE in guinea pig parenchymal tissue are concentration dependent between 0.5 μM (5 mg tension) and 2.5 μM (70 mg tension) with no further contractile effect at 25 and 50 μM. RIA of the sulfido-peptidyl leukotrienes (LTC_4, LTD_4 and LTE_4) released by the parenchymal tissue during peak contraction demonstrated a strong correlation between the contractile effect of HDHE and the leukotriene level in the incubation medium. As much as 6.5 ng/g wet wt of sulfido-leukotriene was measured in the presence of 2.5 μM HDHE and may account in part for the HDHE-induced contraction. Leukotriene C_4 is capable of inducing contractions at this level (20-35 mg tension) which may account for approximately 40-50% of the HDHE contractile response.

The ability of the HDHE positional isomers (17-, 14- and 11-) to stimulate lipoxygenase activity, as compared to A23187, was assessed in lung parenchymal tissue and PMNs. In all cases, the effect of HDHEs on the production of lipoxygenase products was concentration-dependent. For example, as little as 0.2 μM HDHE stimulated leukotriene C_4 production from parenchymal tissue with significant increases in production observed at 2.0 and 20 μM HDHE. The various positional isomers were equipotent in this regard. However, the HDHEs were more efficacious with regard to leukotriene B_4 production; 0.2 μM HDHE significantly stimulated leukotriene production (17>11>14) with marked increases observed at 2.0 and 20 μM HDHE. Other hydroxy fatty acids tested, such as 13-HODE, 12-HETE, 5-HETE and 12-HEPE had little or no effect on leukotriene production. Moreover, appropriate concentrations of the n-3 parent fatty acids 20:5 and 22:6 did not effect leukotriene production with one exception and this was at higher concentrations of 22:6n3 (20 and 50 μM), for which leukotriene production from parenchymal tissue was stimulated.

Human monocytes incubated with ^{3}H-AA produce detectable levels of the radiolabelled cyclooxygenase products 6-keto-$PGF1_{\alpha}$, TXB_2, $PGF_{2\alpha}$ and PGE_2. In the presence of 2 μM 14-HDHE, detectable levels of prostaglandin were not observed. However, a 5-lipoxygenase product, 5-HETE, and a 15-lipoxygenase product, 15-HETE, became detectable. These data suggest 20:4n6 is more susceptible to lipoxygenation as opposed to cyclooxygenation in the presence of HDHE.

As previously reported, low levels of the n-3 hydroxy fatty acids specifically antagonize both the contractile effects of a thromboxane agonist, U46619, and its platelet aggregating effect. It is proposed that the HDHEs may specifically interact with the thromboxane receptors. Preliminary data indicate a direct relationship, albeit weak, between inhibition of U46619 induced platelet aggregation and ^{3}H-U46619 specific binding by 14-HDHE. Interestingly, the parent fatty acid 22:6n3 inhibited both U46619-induced platelet aggregation, as reported by others, and ^{3}H-U46619 binding. It is not clear whether hydroxylation of 22:6n3 significantly alters its ability to interact with the thromboxane receptor. Further determination of the structure-activity relationship of these thromboxane receptor ligands is necessary.

Lipoxygenation of the n-6 and n-3 fatty acids was depressed in platelets obtained from ethanol-dependent rats. Supplementation with a diet rich in 18:2n6 (corn oil) effectively reversed the inhibitory effect of ethanol on 13-HODE production by more than 60%. Similarly, supplementation with a diet rich in 20:5n3 (fish oil) reversed the inhibitory effect of ethanol on 12-HEPE production by more than 80%. Additional methodological development is necessary in order to improve both the separation and quantification of 12-HETE and 14-HDHE in order to make a similar evaluation of their production.

Rat aortic rings obtained from similarly treated animals were tested for their contractile response to U46619. As previously reported, the EC_{50} for U46619 was reduced by as much as 56% in aortic rings obtained from ethanol-dependent rats. A similar effect was observed in aortic rings obtained from fish oil fed animals; the EC_{50} for U46619 decreased by 37%.

Significance to Biomedical Research and the Program of the Institute:

The biological properties of the n-3 hydroxy fatty acids have been further characterized and compared to the n-6 hydroxy fatty acids. Both the content of the parent fatty acids and the capacity to produce their hydroxy metabolites is markedly reduced in tissue obtained from ethanol- dependent animals. Enrichment of the n-6 fatty acids in the diet may counter the effects of ethanol on fatty acid content in peripheral tissues and their subsequent metabolism to biologically active derivatives. It is clear that both smooth muscle contractility and platelet aggregation are significantly altered by nutritive factors. Ethanol and fish oil are known to increase the production of these n-3 hydroxy fatty acids and this may directly contribute to their anti-thrombotic effects. The lipoxygenase stimulating properties of HDHEs in parenchymal tissue, neutrophils and monocytes may indicate a modulatory role in cell-cell interactions that may be of importance in the pulmonary and immune systems.

Proposed Course:

The production and biological activities of lipoxygenase products from other cell types and tissues, especially of excitable ones such as neural and retinal, will be investigated. The Y79 retinoblastoma cell line will be used in this regard. A series of 22:5 positional isomers will be converted to their lipoxygenase products and the biological activity studied in relation to that of the 22:6n3 metabolites. In this way, the question as to whether the essential function of 22:6n3 is due to lipoxygenase products may be addressed. The effect of the hydroxy derivatives of the various polyunsaturated fatty acids upon eicosanoid production and function will also be investigated with a particular focus upon the mechanism by which these compounds antagonize thromboxane-induced smooth muscle contraction and platelet aggregation. A new initiative to study the effects of the novel metabolites of 22:6n3 upon electrophysiological properties of neurons will be made.

Publications:

Karanian JW, Kim HY, Salem Jr N. Effect of nutritive factors on docosahexaenoic acid metabolism to hydroxy-docosahexaenoate (HDHE). In: Racagni G, Brutelli N, Fucado P, eds. Biol Psych, Excerpta Medica Intl Congress Series, 1991;968.

Engler MB, Karanian JW, Salem Jr N. Docosahexaenoic acid (C22:6w3) induced relaxation of the rat aorta, Eur J Pharm 1990;185:223-6.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AA 00284-02 LCS

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Alterations in Lipid Metabolism in the Nervous System by Ethanol

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	H-Y Kim	Research Chemist	LCS, NIAAA
Others:	S. Sawazaki	Visiting Fellow	LCS, NIAAA
	N. Salem, Jr.	Section Chief	LCS, NIAAA

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Clinical Studies

SECTION
Section of Analytical Chemistry

INSTITUTE AND LOCATION
NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL MAN-YEARS: 2.0	PROFESSIONAL: 2.0	OTHER:

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In order to elucidate metabolic functions of polyunsaturated fatty acids and phospholipids in nervous tissues, two aspects were studied: (i) Oxygenation of the two major polyunsaturates in brain, docosahexaenoic acid (22:6n3) and arachidonic acid (20:4n6); (ii) Molecular remodeling of phospholipids in brain tissues. When oxygenation of polyunsaturates by various regions of rat brain, including pituitary, hypothalamus, thalamus, cerebral cortex, cerebellum and pineal body was examined, only the pineal body was found to possess lipoxygenase activity. Both 20:4n6 and 22:6n3 were metabolized by pineal body homogenate, primarily through 12- but also 15-lipoxygenase reaction. The resulting metabolites were also detected in the pineal body lipid extract, possibly indicating endogenous production of these compounds. 15-lipoxygenase products were the major ones present in the lipid extract of pineal body, suggesting compartmentalization of the LO enzymes. Preliminary results indicated that the level of 17-hydroxy 22:6n3 in the pineal bodies of ethanol treated animals was increased by 50%. In order to understand the means by which 22:6n3 or 20:4n6 fatty acid is concentrated in certain lipid classes in brain, phospholipase (PLA) and phosphatidylserine (PS) decarboxylase were examined. Myelin, synaptosomal membranes and mitochondrial fractions were prepared, and among these only the mitochondrial fraction contained significant PLA2 and PS decarboxylase activities. Furthermore, in rats exposed to ethanol inhalation for 14 days, an increase in PLA2 activity of approximately 70% was observed in the brain mitochondrial fraction. Also, alcohol-induced losses of 20:4n6 and 22:6n3 was observed in a non-extractable fraction which was associated with membrane proteins.

Project Description:

Investigators:

H-Y Kim	Research Chemist	LCS, NIAAA
N. Salem, Jr.	Section Chief	LCS, NIAAA
S. Sawazaki	Visiting Fellow	LCS, NIAAA

Objectives:

(i) To delineate the functions of polyunsaturated fatty acids and their metabolites in the nervous system, and (ii) to describe the alterations in lipid composition and metabolism caused by ethanol and the resulting changes in these functions.

Methods Employed:

Preparation of Hydroxy and Hydroperoxy Derivatives

Docosahexaenoic (22:6n3), arachidonic (20:4n6), eicosapentaenoic (20:5n3), linoleic (18:2n6) and linolenic (18:3n3) acids were autooxidized by exposing a thin film of the lipid spread on a round bottomed flask to oxygen overnight. The 15- and 12-lipoxygenase products were prepared by incubating these fatty acids with soybean lipoxygenase and platelets, respectively. The 14-hydroperoxy-22:6n3 as well as epoxy, hydroxy compounds were prepared by the incubation of partially purified porcine leukocyte 12-lipoxygenase with 22:6n3. Oxygenated polyunsaturates were extracted with dichloromethane (containing the antioxidant BHT) after acidification to pH 3 with formic acid. They were characterized using thermospray LC/MS for the hydroperoxy derivatives and by GC/MS after reduction, purification and derivatization to the methyl ester-TMS ethers.

Preparation of Hydroxy Fatty Acids from Rat Brain

Male Sprague Dawley rats (250-300 g) were used and kept under controlled lighting from 0700 to 1900 and fed by free access to standard rat chow diet and water. They were harvested between 1000 to 1100 in order to control for diurnal effects. Rats (200-300 g) were anesthetized with an intraperitoneal injection of chloral hydrate and the brain was perfused with oxygen-saturated physiological saline containing heparin. The brain was rapidly removed and dissected into seven different regions (cerebral cortex, cerebellum, hypothalamus, thalamus, caudate, pituitary, and pineal body) using a razor blade and immediately placed in ice cold phosphate buffered saline (PBS, pH 7.4). The brains were then either sliced in ice-cold PBS buffer containing 35 mM glucose or homogenized in 50 mM Tris buffer (pH 7.4). Brain slices (about 100 mg) or 1 mL of a 10% brain homogenate were incubated with 35 μM of 1-[^{14}C]-22:6n3 or 20:4n6 in the presence of calcium (2 mM) for 30 min at 37°C. The pineal bodies were homogenized and diluted in oxygen-saturated PBS to a concentration of 3 mg/ml prior to incubation. Various inhibitors, stimulators or neurotransmitters were added as appropriate. Control incubations were also carried out on rat brain slices or homogenate that were boiled for 20 min.

Reactions were terminated by acidification to pH 3 using 15% formic acid and the products were extracted with dichloromethane followed by ethyl acetate.

Analysis of Oxygenated Products Formed by Rat Brain

Various positional isomers of rat brain hydroxy products were separated on an Axxiom-ODS column (5µ, 4.6 mm x 25 cm) using an HP-1090 HPLC system equipped with a diode array UV detector. A radioactivity flow detector was connected in series for the analyses of radioactive metabolites. The mobile phase consisted of 0.1 M ammonium acetate and methanol (40:60 to 15:85 in 50 min) with a flow rate of 0.8 mL/min. Thermospray LC/MS analysis was performed using a Finnigan TSQ-70 triple quadrupole mass spectrometer. GC/MS/EI analysis was carried out using an HP-5970 MSD after methylation and trimethylsilylation using an HP-1 methyl silicon column with oven, injector and transfer line temperature of 220, 230 and 250°C. Stereochemical analyses were performed using a dinitrobenzylphenylglycine column (5µ, 4.6 mm x 25 cm) with a mobile phase of hexane:2-propanol: acetonitrile (497.5:2.5:0.5) and a flow rate of 0.8 mL/min.

Measurement of Endogenous Hydroxy Fatty Acids Produced in Pineal Body

Pineals obtained from control and alcoholic rats were homogenized in methanol containing 50 µg/ml BHT, and extracted according to the Bligh and Dyer procedure in the presence of deuterated internal standards. After solid phase extraction using C-18 cartridges according to Powell, GC/MS/NCI analysis was carried out using an HP- 5989 mass spectrometer after pentafluorobenzyl esterification, hydrogenation and TMS derivatization. An SPB-1 column (0.25 mm x 30 m, 0.25µ) was used with the oven temperature programmed from 230 to 320°C at the rate of 5°C/min.

Measurement of Leukotriene B_4

Production of Leukotriene B_4 was measured using thermospray LC/MS in the presence of $^{13}C_2$-LTB_4 as an internal standard, after pentafluorobenzyl derivatization. Derivatized metabolites were injected onto an ODS column (4.6 mm x 7.5 cm, 3 µ) and separated using a mobile phase of 0.1M ammonium acetate/acetonitrile (40/60) at a flow rate of 1 mL/min. They were detected in a on-line fashion by a negative ion mode thermospray technique in either a discharge-on or filament-on condition. The source and vaporizer temperatures were maintained at 250 and 75°C, respectively.

Preparation of Brain Subcellular Fractions

Forebrains (without the thalamus or hypothalamus) of ethanol treated or control rats were removed and homogenized in 9 volumes of 10% (w/w) sucrose using a motor driven teflon-in-glass homogenizer. The homogenate was centrifuged at 800 x g for 20 min and the resulting supernatant was centrifuged at 9,000 x g for a further 20 min using a Beckman JA17 centrifuge. The pellet was washed once with 10% sucrose for preparation of a crude synaptosomal fraction. In order to lyse the synaptosomes, this pellet was resuspended in 5 mM Tris-HCl buffer and incubated on ice for 30 min prior to homogenization by hand using six strokes and a teflon-in-glass homogenizer. An appropriate volume of 48% (w/w) sucrose was added to yield a 34% sucrose solution, and 28.5% (w/w) sucrose was overlaid. A small volume of 10% sucrose was overlaid on the top of the gradient and centrifuged at 60,000 x g for 110 min at 4°C using a Beckman L8-70 ultracentrifuge. Three fractions including myelin, synaptosomal membrane and mitochondrial fractions were collected.

Fatty Acid Analysis

Various brain tissues and subcellular fractions were extracted according to Bligh an Dyer and transmethylated using BF_3-methanol (14% w/v) at 100°C for 2 hrs according to Morrison and Smith. After extraction three times with hexane, the extracts were dried under nitrogen and redissolved in hexane. Fatty acid methyl esters were injected into an HP 5880A gas chromatograph equipped with a flame ionization detector. A DB-FFAP capillary column (0.25 mm i.d. x 30 m, 0.25 μ film thickness) was used with an oven temperature programmed from 130 to 175°C at 4°C/min and then to 210°C at 1°C/min. Hydrogen was used as carrier gas with a linear velocity of 54 cm/sec.

Major Findings:

Hydroxylation of Polyunsaturates by Rat Brain

When 22:6n3 was incubated with rat brain homogenate, all of the ten possible positional isomers of monohydroxy-22:6n3 (HDHE) were identified in the incubation mixture. From their racemic stereochemical distribution and inhibitor profile, we have determined that they were not produced by lipoxygenation but by peroxidation. With the exception of the pineal body, other brain regions did not exhibit any measurable lipoxygenase activity. When pineal body homogenate was incubated with 20:4n6 or 22:6n3, oxygenated products were produced in a time and dose dependent manner. Analyses of their structures by thermospray LC/MS and GC/MS/EI indicated that 12- and 15-HETE were produced from 20:4n6 and 14- and 17-HDHE from 22:6n3, with the 12-lipoxygenase products being the primary metabolites. The 15- lipoxygenase activity toward 22:6n3 appeared to be higher than that for 20:4n6, as the production of 17-HDHE is 4 times higher than that of 15-HETE while 12-lipoxygenase products of the two substrates were formed at a comparable level. Production of these hydroxy compounds by brain was not prevented by cyclooxygenase or cytochrome P-450 dependent monooxygenase inhibitors, but production was reduced by lipoxygenase inhibitors and by boiling. Stereochemical analysis by chiral phase HPLC revealed that they were in the S form. These data suggested that they were formed by the lipoxygenase mechanism.

In addition to 12-HETE, hepoxilin B_3 was also produced by the pineal body in variable amounts. This epoxy, hydroxy derivative of 20:4n6 is known to be formed through a unique intramolecular rearrangement of the 12-hydroperoxy 20:4n6, which is the initial product of 12- lipoxygenase reaction. A similar compound was produced in small amounts by the pineal body homogenate from 22:6n3. This minor compound was considered to be a hepoxilin-like compound, as this peak showed no UV absorbance at 235 nor 280 nm, and coeluted in the reversed phase chromatographic system with the epoxy, hydroxy compound produced from the reaction mixture of 22:6n3 and porcine leukocyte 12-lipoxygenase. Upon incubation of 22:6n3 with partially purified porcine leukocyte enzyme, 14-hydroperoxy and hydroxy-22:6n3 were formed. In addition, 13,14-epoxy, hydroxy-22:5n3 was also produced as evidenced by its thermospray spectrum which contained fragment peaks resulting from the characteristic cleavage of the epoxidated C-C bond. This epoxy, hydroxy compound was presumably produced from 14-hydroperoxy 22:6n3, and coeluted with the hepoxilin-like compound produced by the pineal body.

In order to measure the endogenous production of these lipoxygenase products, rat brains were perfused, the pineal was removed and a lipid extract made by the Bligh-Dyer procedure in the presence of an antioxidant and deuterated internal standards. They were then separated by solid phase extraction, hydrogenated, derivatized to the pentafluorobenzyl ester, trimethylsilyl ethers and analyzed by NCI mass spectrometry. The 12- and 15-HETE as well as 14- and 17-hydroxy 22:6n3 were detected from the lipid extract in pineal bodies, indicating that these products were formed endogenously. However, in contrast to the case of incubation with exogenous substrates, the major compounds formed were not the 12-lipoxygenase products but rather the 15-lipoxygenase products. These results suggest that these two enzymes are compartmentalized differently, for instance, 12-lipoxygenase may be located near the vasculature, where its metabolites may be easily washed off by perfusion, whereas 15-lipoxygenase may be localized in the parenchyma. Preliminary results indicated that the level of 17-hydroxy 22:6n3 in the pineal bodies of ethanol treated animals was increased by 50%, and the possible involvement of this compound in pineal functions in relation to ethanol treatment is now under investigation. When the production of leukotrienes was measured using a newly developed thermospray LC/MS method, no LTB_4 production was observed (0.5 ng detectable) in various regions of brain, while the non-perfused pituitary produced nanogram levels of LTB_4. These results indicated that the 5-lipoxygenase activity previously reported might have been the result of blood cell contamination.

Phospholipid Metabolism

Phospholipid remodeling studies using deuterated or radioactive phospholipid molecular species and thermospray mass spectrometry are now in progress. When perfused brain homogenate was incubated with ^{14}C-di-18:1-PS, decarboxylation to di-18:1-PE was observed. No decarboxylation was observed in a boiled control. When subcellular fractions including myelin, synaptosomal membrane and mitochondria were prepared using a sucrose gradient method, the mitochondrial fraction contained most of the metabolic activity with respect to phospholipase A_2 and decarboxylation. Preliminary results indicated an increase in phospholipase A_2 activity of the brain mitochondrial fraction obtained from rats receiving ethanol inhalation for 14 days. The fatty acid profile of these subcellular fractions was not grossly altered by ethanol treatment, however, loss of 20:4n6 was observed in protein pellets of all fractions, particularly in the mitochondrial fraction, suggesting that selective losses of polyunsaturates occur in the vicinity of proteins. In protein pellets of the mitochondrial fraction, significant loss of 22:6n3 was also observed after ethanol treatment.

Significance to Biomedical Research and the Program of the Institute:

It has been proposed that an important mechanism underlying many of the behavioral effects of ethanol is its capacity to alter polyunsaturate metabolism through cyclooxygenase and lipoxygenase pathways. In this project, we focused upon brain lipoxygenase as one possible target for ethanol action in the CNS and attempted to extend our knowledge of this pathway by exploring new substrates of particular relevance to the excitable tissues. Our studies indicated that, contrary to the prevailing belief, peroxidation and not lipoxygenation is the major mechanism for observed oxidation of 22:6n3 and 20:4n6 in brain. Lipoxygenase activity was not observed in the cerebrum, cerebellum, thalamus, hypothalamus, caudate and pituitary. However, in the pineal body homogenate both 12- and 15-lipoxygenase activity were found for 20:4n6 and 22:6n3 with the 12-lipoxygenase activity predominating. Endogenous production of these lipoxygenase products was also observed, however, the 15-lipoxygenase products were the principal ones present. These results together with the high content of 22:6n3 in the pineal indicated that 15-lipoxygenase metabolites of 22:6n3 may play an important role in pineal function.

Proposed Course:

We will continue our characterization of metabolic pathways of polyunsaturates in the nervous system. Our studies of brain peroxidation will be extended to an in vivo paradigm in rat brain. Lipoxygenation in pineal and the biological effect of lipoxygenase metabolites in relation to ethanol will be further investigated. Peroxidized phospholipid will be investigated in relation to its formation in target organs and their elaboration by ethanol. Investigation of the selective metabolism of phospholipid molecular species will continue using radioactive precursors or deuterated lipid standards and mass spectrometry. Alterations in composition and metabolic functions of polyunsaturates by ethanol in defined brain subcellular fractions will continue. Developmental efforts for quantification of phospholipid molecular species and leukotrienes using thermospray LC/MS will continue.

Publications:

Kim HY, Shingu T, Karanian JW, Salem N Jr. Stereochemical analysis of hydroxylated docosahexaenoates produced by human platelets and rat brain homogenate, Prostaglandins 1990;40:473-90.

Kim HY, Karanian JW, Salem N Jr. Formation of 15-lipoxygenase products from docosahexaenoic acid by human platelets, Prostaglandins 1990;40:539-49.

Kim HY, Salem N Jr. Separation of lipid classes by solid phase extraction, J Lipid Res 1990;31:2285-9.

Kim HY, Sawazaki S, Salem N Jr. Lipoxygenation in rat brain? Biochem Biophys Res Comm, 1991;2:729-34.

Moore SA, Giordano MJ, Kim HY, Salem N Jr, Spector AA. Brain microvessel 12-hydroxyeicosatetraenoic acid is the (S) enantiomer and is lipoxygenase derived, J Neurochem, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AA 00235-09 LCS

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Nutritional Effects on Essential Fatty Acid Composition

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	N. Salem, Jr.	Section Chief	LCS, NIAAA
Others:	M. Bossant	Visiting Fellow	LCS, NIAAA
	Y. Denkins	NRC Fellow	LCS, NIAAA
	H-Y. Kim	Research Chemist	LCS, NIAAA
	C. Niebylski	NRC Fellow	LCS, NIAAA

COOPERATING UNITS *(if any)*
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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.8	2.8	1.0

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

It is our hypothesis that alcohol abuse leads to the depletion of polyunsaturated lipids from many tissues and leads to altered metabolism of eicosanoids. Such changes in the liver as well as in other organs and tissues may contribute to the pathological changes associated with alcoholism. Losses in arachidonic acid (20:4n6) and other n-6 fatty acids have been observed in our studies of alcohol exposed rat tissues as well as in the blood cells of alcoholics. This led to the question as to whether it was possible to replace these essential fatty acids by dietary manipulation. Animal studies have indicated that supplying the n-6 precursor, 18:3n6 in the diet can partially prevent the alcohol-induced loss of liver 20:4n6 when compared to diets high in 18:1n9 or long chain n-3 fatty acids. A clinical study of alcoholics during withdrawal has indicated that the composition of polyunsaturates is altered in a complex fashion in blood cells. Platelets exhibited a loss of a 20:4n6 species with increases in several non-essential phospholipid species in alcoholics at admittance. Most of these changes normalized after 4 weeks of withdrawal. In the erythrocyte, there was a loss of 18:2n6 and an increase in the non-essential species 18:0 and 18:1n9. None of the species that were abnormal at the beginning of alcohol withdrawal had normalized in the erythrocyte by 4 weeks. Arachidonyl species increased in PE while decreasing in PS. There was a generalized increase in phospholipid molecular species asymmetry in the erythrocyte membrane in alcoholics.

Previous title: Nutritional Effects on Lipid Composition

Project Description:

Investigators:

N. Salem, Jr.	Section Chief	LCS, NIAAA
M. Bossant	Visiting Fellow	LCS, NIAAA
Y. Denkins	NRC Fellow	LCS, NIAAA
H-Y. Kim	Research Chemist	LCS, NIAAA
H. Knapp		Univ. of Iowa
C. Niebylski	NRC Fellow	LCS, NIAAA
A. Yoffe	Chemist	LCS, NIAAA

Objectives:

To (i) test the hypothesis that ethanol exposure leads to alterations in lipid and fatty acid composition and, in particular, a loss of polyunsaturates from membrane phospholipids; (ii) determine the consequences of essential fatty acid deficiency for the organism with particular reference to nervous system function; (iii) evaluate the biological functions of highly unsaturated omega-3 fatty acids in cell membranes; (iv) determine whether supplementation of essential fatty acids in the diet may be used to reverse or prevent losses in membrane polyunsaturates and thus be of therapeutic value to alcoholics.

Methods Employed:

Lipid Extraction

Lipids were extracted from brain, liver, heart, aorta, platelets and plasma according to the method of Bligh and Dyer using chloroform-methanol-water in the ratios of 1:2:0.8. A small amount (50 mg) of the antioxidant, BHT was added to each sample. Tissues were homogenized using a Polytron (Brinkman) cavitation type instrument and then one volume of chloroform and water was added to partition the mixture into two phases. The lipid extraction method of Reed was used in some cases for extraction of erythrocytes. When necessary, phospholipid classes were separated by thin layer chromatography using the method of Rouser and coworkers; the solvent system employed was chloroform-acetone-methanol-acetic acid-water (5:2:1:1:0.5). The gel containing individual phospholipid classes was scraped off of the plates and directly transmethylated, as above.

Fatty Acid Analysis

Portions of these total lipid extracts were evaporated under a stream of nitrogen and transmethylated with 14 wt% BF_3 in methanol for 2 hrs at 100°C using a modification of the method of Morrison and Smith. Hexane was used in place of benzene as a cosolvent in order to minimize exposure of lab personnel to the latter agent. It was observed in studies of fish oil, plasma and brain samples that this substitution produced no observable losses in any of the component fatty acids. The methyl ester derivatives were extracted into hexane and evaporated under nitrogen for injection into an HP-5880A gas chromatograph equipped with a flame ionization detector. A 30 m x 0.25 mm ID fused silica capillary column (J & W Scientific) was used with hydrogen as carrier gas at a linear velocity of 50 cm/sec. Injector, detector and initial column temperatures were 240, 240 and 130°C, respectively. The oven temperature rose at a rate of 4°C/min until 175°C and then at a rate of 1°C/min until 210°C was reached. Finally, the oven temperature was increased at 30°C/min until 240°C was reached and the remainder of the run was at this temperature. Samples were injected by means of a 7673A autosampler with a split ratio of 5:1. Fatty acids were identified by comparison to primary and secondary standards and by electron impact mass spectrometry on an HP-5970B mass selective detector.

HPLC Analysis of Molecular Species Composition and Asymmetry

Cell aminophospholipids were labeled with 2,4,6-trinitrobenzenesulfonic acid (TNBS) by a modification of the procedure of Marinetti and Love. One ml of packed RBC were reacted with 15 ml of ice-cold freshly prepared TNBS solution (2 mM final concentration) containing 120 mM $NaHCO_3$, 40 mM NaCl and 4.4 mM glucose, pH 8.3. They were incubated under non-penetrating conditions, i.e., for 30 min at 0°C using gentle agitation on an orbital shaker. Excess reagent was removed by centrifuging at 1000 x g for 10 min at 4°C. The cells were then washed twice with ice-cold buffer (pH 7.4); in the first wash, 0.5% fatty acid free BSA was added to prevent further TNBS reaction. Lipids were extracted according to the method of Reed and a portion spotted on a silica gel plate for phospholipid class separation. TNP-PE derivatives, as visualized by their yellow color, were separated from unreacted PE and PS and from other phospholipid classes using the solvent system of chloroform : acetone : methanol (containing 100 mg BHT) : acetic acid : water (5:2:1:1:0.5, v/v). The plates were dried under nitrogen and the yellow bands were scraped and extracted twice with 3 ml of methanol containing 50 mg of BHT. The extracts were centrifuged at 3000 RPM at 4°C for 20 min in order to remove fine particles of silica gel. After evaporation under nitrogen, the extracts were dissolved in methanol. These TNP-PE derivatives corresponded to the PE of the outer leaflet of the plasma membrane. They were subfractionated into molecular species of TNP-PE and quantified by HPLC as described below. Portions of the total lipid extract were reacted with TNBS in a basic/organic solvent system so that all species of aminophospholipid were labelled and then separated and processed as described above.

Separation of molecular species of TNP-PE and TNP-PS was performed on an HP 1090 liquid chromatograph equipped with an HP 1040 diode array detector and an HP 79995A analytical workstation. An Axxichrom ODS column and a mobile phase of 10 mM ammonium acetate, pH 5.0 and methanol were employed at a flow rate of 1 ml/min and a column temperature of 4°C. The analysis began with a 5 min isocratic elution at 84% methanol, followed by a linear increase to 87% methanol in 15 min; this proportion was then maintained for 40 min. TNP-PE and PS were quantified by their absorbance at 338 and 342 nm, respectively. A linear relationship of peak area to molar amounts was found for the various TNP molecular species standards. They were separated on the RP-HPLC column according to differences in the chain length and degree of unsaturation in their fatty acid components. Since isocratic conditions were used during most of the analysis period for the TNP-PE species, a relationship between elution times and the fatty acid composition at the sn-1 and sn-2 positions was found for the various TNP-PE molecular species.

Synaptosome preparation

Synaptosomal membranes were prepared by the method of Cottman et al. Briefly, the forebrains of male, Sprague-Dawley rats (150-200 g) were removed rapidly and homogenized in an ice cold 0.32 M sucrose-5 mM HEPES buffer containing 1 mM EDTA, pH 7.5 so as to make a 20% w/v mixture. The crude nuclear pellet was removed by centrifugation at 1100 x g for 5 min. The resulting supernatant was centrifuged at 17000 x g for 10 min to obtain a crude mitochondrial pellet. This pellet was resuspended and layered onto a discontinuous Ficoll-sucrose gradient. High speed centrifugation (64000 x g, 45 min) yielded a synaptosomal fraction at the 7.5 - 13% Ficoll-sucrose interface. The fraction was washed twice and stored in 5 mM HEPES buffer, pH 6.8 at 5°C until used.

Chemical Cross-linking of Synaptosomes

An aliquot of the synaptosomal preparation (1-4 mg protein) was reacted with 100 mM difluorodinitrobenzene (DFDNB) for 30-60 min in 5 mM HEPES buffer at pH 8.5 and 37°C. The protein was pelleted and the non-crosslinked lipid extracted several times using the method of Bligh and Dyer. The protein residue was dried under a stream of nitrogen and transmethylated as described above.

Fluorescence

A state-of-the-art fluorimeter (PTI model LS-100) capable of measuring absorption/emission spectra, fluorescence and phosphorescence lifetimes and steady state and time-resolved polarization was installed and its functions verified.

Dietary Studies

Juvenile rats (about 70 g) were first acclimated to eating a powdered diet for 5-7 days prior to presentation of the experimental diets. The diet used for this purpose was standard 10% rat chow pellets that had been pulverized or an olive oil based diet. The diets were then switched to a purified diet similar in composition to the AIN-76A formulation except that the fat content was raised to 10 wt%, sucrose was reduced to 50 wt% and replaced with corn starch, Teklad vitamin mix (#40060) was used in place of the AIN mix, and calcium carbonate was increased to 0.43 wt%. The caloric density of the diets was adjusted by a factor of 1.07 in order to match that of the AIN recommended diet. The stock diet was made up with 1 wt% corn oil in order to supply the essential fatty acid, 18:2n3. The other oils, menhaden, corn, olive and borage oil, were added as needed to make up the remaining 9 wt% and stored at 4°C under nitrogen and for not more than 2 weeks in order to prevent oxidation of the unsaturated acids. All oils contained both TBHQ (0.02 wt%) and vitamin E (0.3 wt%) and were balanced in this regard.

Major Findings:

A clinical study involving 55 alcoholic inpatients and 33 normal volunteers was completed during this reporting period. Fatty acid analyses and, in some cases, aminophospholipid molecular species quantitation was carried out on the erythrocytes and platelets of controls and alcoholics at admittance and at discharge. Clinical blood chemistries and cell counts indicated that alcoholics had significant elevations in phosphorus, the liver enzymes SGPT, SGOT and GGTP, cholesterol, triglycerides, mean corpuscular volume and white blood cell count.

In the erythrocyte, alcoholics had a decrease in 18:2n6 and an increase in 20:3n6 in both phosphatidylcholine (PC) and phosphatidylethanolamine (PE). The 18:2n6 decline in the alcoholics was confirmed with the molecular species analysis in that total and cell surface 18:1,18:2n6-PE was significantly decreased. There was a concomitant increase in the non-essential species 18:0,18:1n9-PE in the cell surface fraction of PE in alcoholics. The 20:4n6 species alterations were more complex. Both the 18:0-alkenyl, 20:4n6 in the whole cell and the 16:0, 20:4n6-PE species in the cell surface fraction increased in the alcoholic. However, the 16:0, 20:4n6 species of PS decreased from a normal volunteer level of 6.7 to 4.9% in the alcoholics at admittance. For 13 of the 18 PE molecular species quantified, there was an increase in the symmetry index in the alcoholics when the cell surface and total plasma membrane PE species distributions were considered. Also, there was a strong correlation between the 18:0,20:4n6-PE in either the total membrane or on the cell surface with the quantity and frequency of alcohol intake during the 6 month period prior to hospitalization as well as one with the GGTP value. There was a negative correlation between this peak and both age and cholesterol level.

Alcoholics were reevaluated after 3-4 weeks of alcohol abstinence while hospitalized on our ward. During this period, erythrocyte PE analysis indicated that the 20:3n6 normalized but the 18:2n6 continued to decline. It was also of interest that 20:4n6 declined significantly from that of the level of these patients at admittance. PE species analysis confirmed that 18:1,18:2n6 remained low in the patients at discharge. In fact, none of the erythrocyte species that were abnormal at admittance normalized after 3-4 weeks of hospitalization. In PC, both 16:1n7 and 18:1n9 normalized at discharge. Much of the decline in 18:2n6 was also reversed but the level was still below that of normal volunteers. In addition, at discharge, the 20:3n6 and 20:5n3 continued to increase and thus remained abnormal.

In the platelet, alcoholics at admittance had decreases in 18:0,20:4n6 and increases in 18:1,18:1n9; 18:0,18:2n6 and 18:0alkenyl,22:5 PE species. After 4 weeks of hospitalization, none of these species were significantly different than normal volunteer values with the exception of 18:1,18:1n9 which remained abnormally high. The species containing 18:0 alkenyl at the sn-1 coupled with either 22:4n6 or 22:5n3 at the sn-2 position were both decreased at discharge in the alcoholics in comparison to their values at admittance. In the platelet PS, further differences were noted when the alcoholics were compared at admittance versus at discharge. There was a decrease in 16:0,22:6n3 and

18:0,22:6n3 after the period of hospitalization. The disaturated species, 16:0,18:0 was decreased at admittance but normalized at discharge.

In this clinical study, nutritional history information was taken and food intake analysis was also performed on a subset of the patients. The food data was converted to nutrient information which includes individual fatty acid and vitamin intake and a data base was created so that these variables may be correlated with the lipid compositional data and clinical variables.

A dietary experiment was performed in order to test the influence of dietary fatty acids upon the ability of ethanol to alter the fatty acyl profiles of rat liver, brain, platelet and erythrocytes. Animals were divided into six groups and given either an olive, borage or menhaden oil based diet either alone or in combination with ethanol inhalation for 7 days. The animals were fed on their respective diets for two weeks prior to being placed in the inhalation chambers for alcohol exposure. The average blood alcohol levels in these animals was about 170 mg%. In all tissues the fatty acyl profiles of the total lipid extract reflected the acyl composition of the diet. In the olive oil diet, 18:1n9 and 20:3n9 were generally increased. In the borage diet, the n-6 polyunsaturates were increased at the expense of the n-3 fatty acids. Conversely, the menhaden oil diet led to an increase in the n-3 polyunsaturates at the expense of the n-6 fatty acids. The liver was the most drastically affected compartment of those tested by these diets although there were also substantial changes in the blood cell acyl distributions; the brain total lipid extracts were the least altered. For example, in the olive, borage and menhaden oil diets, the 20:4n6 levels were 12.4, 23.6 and 5.3% whereas the 22:6n3 levels were 2.7, 1.3 and 14.7%, respectively. The corresponding 18:1n9 levels were 29.7, 9.9 and 9.3% in these diets. It was of importance that the extent of the essential fatty acid deficiency caused by ethanol inhalation was dependent upon the type of unsaturated fatty acid in the diet. For example, the liver 20:4n6 level was decreased by 44 and 36% in the olive and fish oil groups in comparison to their respective controls, however, the borage oil diet produced an only 26% decline in this essential fatty acid after alcohol exposure for 7 days. This may have been expected since borage oil contains 18:3n6 and this bypasses the delta-6 desaturase, one of the proposed targets for alcohol action. There was also an increase in 20:3n6 and 22:4n6 in the livers of the ethanol exposed borage oil-fed group. Another example of a case in which dietary modification could lead to qualitatively different responses of lipid composition was that of 22:6n3. In the olive oil diet, the liver total lipid extract 22:6n3 decreased by about 25% in the alcohol exposed group whereas in the other two diets it was increased. In the borage oil diet, there was actually a 65% increase in 22:6n3 in the total lipid extract after alcohol exposure. In the platelet, erythrocyte and brain, the 22:6n3 level was generally unchanged or increased by alcohol exposure. In most cases, this was also true of the total n-3 fatty acids and the n-3/ n-6 ratio. However, these analyses of the whole organ total lipid extracts should not be interpreted to preclude the loss of n-3 fatty acid nor of 22:6n3 in particular in specific subcellular fractions, lipid classes or other compartments.

Biochemical and biophysical studies were initiated in order to define the structural and regulatory role of polyunsaturated phospholipids in cell membranes. Crosslinking of the synaptosomal membranes indicated in our initial experiments that there was an increased proportion of 20:4n6 and 22:6n3 in the protein fraction when compared to a non-crosslinked control. There was a corresponding decrease in 18:0, 18:1n9 and 20:3n6 in the protein fraction. This indicated a preferential association of certain polyunsaturated species of aminophospholipid with synaptosomal membrane proteins.

Fluorescence studies have established that the instrument is capable of detecting femtomolar concentrations of some probes and is capable at this level of providing reproducible anisotropy data. A variety of phospholipid molecular species are being prepared in which the head group, sn-1 and sn-2 position fatty acids are systematically varied. In this way, artificial membranes can be prepared and only one of these features varied in order to determine the biophysical consequences. Fluorescent phospholipids have been obtained of different lipid classes and containing different fluorophores in place of fatty acids. This will make possible direct comparisons of the physical states of different lipid classes and species. Finally, phospholipids containing various 22:5 positional isomers are being prepared in order to compare their membrane properties to those containing 22:6n3. An ongoing effort has been made to evaluate the purity of a variety of fatty acids and phospholipid species and to prepare or purify several that are not commercially available.

Significance to Biomedical Research and the Program of the Institute:

These dietary studies have established that alcohol can produce abnormalities in lipid compositions in various tissues in both animal models of alcohol abuse and in alcoholic patients. New methods of membrane analysis are being developed in order to probe the mechanisms underlying the adverse effects of polyunsaturate losses. Dietary studies have also clearly shown that the composition of these tissues is potently influenced by the type of fats in the diet. Furthermore, supplementation of the diet with n-6 polyunsaturated fat can prevent the alcohol induced alterations in platelets and lessen its effect in the liver. The losses of polyunsaturates along with the resulting changes in their eicosanoid metabolites may provide a mechanism for many of the pathological changes observed in the organ systems of alcoholics. Dietary therapy may therefore be indicated in order to restore pools of tissue polyunsaturates in recently withdrawn alcoholics.

Proposed Course:

Further dietary studies are needed in order to delineate both the restorative and preventive effects of various classes of dietary fatty acids in alcohol exposed animals. Brain, liver and plasma composition must be analyzed in much greater detail in alcohol exposed animals on n-3 and n-6 supplemented diets as alterations in n-3 metabolism may be found. Future studies will attempt to extend our dietary protocols developed in the laboratory to determine if they can more quickly and effectively restore a proper level of polyunsaturated lipids in the tissues of alcoholics. It will be necessary to understand the dose-response relationship of dietary fat type and the reversal of ethanol-induced lipid compositional effects.

The high-level changes in nervous system function due to docosahexaenoic acid deficiency will be evaluated through the development of animal models. The alterations in protein-lipid interactions and in membrane biophysical properties with respect to polyunsaturated species will be investigated in an attempt to understand these organ level changes.

Publications:

Salem Jr N, Carlson SE. Growth and development in infants, World Rev Nutr Diet 1991;66:20-5.

Simopoulos AP, N Salem Jr. Fatty acid content and w-6 to w-3 ratio of chicken egg yolk: differences between free range Greek chicken egg and US supermarket egg, N Eng J Med 1989:ii:1412.

Engler MM, Karanian JW, Salem Jr N. Ethanol inhalation and dietary n-6, n-3 and n-9 fatty acids in the rat: effect on platelet and aortic fatty acid composition, Alcoholism 1991;15:483-8.

Hullin F, Bossant MJ, Salem Jr N. Aminophospholipid molecular species asymmetry in the human erythrocyte plasma membrane, Biochim Biophys Acta 1991;1061:15-25.

Engler MM, Karanian JW, Salem Jr N. Influence of dietary polyunsaturated fatty acids on aortic and platelet fatty acid composition in the rat, Nutrition Research, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AA 00262-08 LCS

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Desaturation of Essential Fatty Acids Using Stable Isotope/Mass Spectrometry

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	N. Salem, Jr.	Section Chief	LCS, NIAAA
Other:	R. Pawlosky	Staff Fellow	LCS, NIAAA

COOPERATING UNITS *(if any)*
None

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Laboratory of Clinical Studies

SECTION
Section of Analytical Chemistry

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Desaturation is a necessary step in the modification of essential fatty acids linoleic and linolenic acids to arachidonic and docosahexaenoic acid, respectively. The levels of these fatty acids are lowered by ethanol consumption in blood cells, liver and brain. The mechanisms underlying the disruption of essential fatty acid levels in these tissues is largely unknown. One possible explanation for this alcohol-induced deficiency is inhibition of elongation/desaturation of 18-carbon essential fatty acids by alcohol. A stable isotope gas chromatography/mass spectrometry (GC/MS) method has been developed that allows for the in vivo examination of these processes. This technique has improved the sensitivity of the method by several orders of magnitude and has made possible non-invasive metabolic studies in large animals. Studies in rats, cats and rhesus monkeys have indicated that all mammals have elongation and desaturation capability and that it may be monitored in the blood supply. This approach has made possible human trials in which we will investigate the effects of alcohol abuse and withdrawal as well as diet on desaturation and chain elongation.

Previous title: Characterization of Fatty Acid Metabolites by GC/MS

Project Description:

Investigators:

N. Salem	Section Chief	LCS, NIAAA
R. Pawlosky	Staff Fellow	LCS, NIAAA

Objectives:

The objectives of this study are: i) to develop a specific and sensitive assay for measuring trace levels of deuterated fatty acid metabolites; ii) to examine dietary factors which may regulate desaturase activity in vivo; iii) to investigate the generality of desaturase enzyme expression in mammals; iv) to investigate the role of ethanol in altering the capacity of an organism to desaturate dietary linoleic and linolenic acids to the 20- and 22- carbon end products in the omega-3 and omega-6 fatty acid pathways.

Methods Employed:

In Vitro Incubation Procedure

Liver homogenates from adult male rats were prepared. The tissue was homogenized in 0.1 M phosphate buffer at pH 7.4 with 2 mM reduced glutathione and 25 mM sucrose. Incubation mixtures contained 0.2 mM Co-A, 2 mM MgC12, 2 mM ATP, 0.5 mM NADH, 0.5 mM NADPH, 0.5 mM malonyl-CoA, 25 μM fatty acid and 50 mM BSA. Protein (4-7 mg) was added to the incubation mixture in a final volume of 2 ml. Samples were incubated at 37°C for 15 min in a shaking water bath.

Extraction-derivatization

Reactions were stopped by the addition of 4 ml of 10% methanolic KOH. Samples were extracted with diethyl ether-petroleum ether 1:1 (v:v). The fatty acids were derivatized to the PFB esters with pentafluorobenzyl bromide-diisopropylamine-acetonitrile (1:10: 1000). The solvent was evaporated and the residue dissolved in 1 ml of hexane for GC/MS analysis.

GC/MS Analysis

Fatty acid PFB esters were run on a 30 m DB-FFAP capillary column using splitless injection with an oven temperature program of 80-185°@20°/min, 185-240°@10°/min and held for 30 min. The source temperature was 150° and pressure (99.99%) was 4 X 10^4 torr. Fatty acids were analyzed as the M-PFB ion in SIM on a Hewlett Packard 5989 mass spectrometer operated in the NCI mode. PFB derivatives of the fatty acids were characterized with regard to their elution order and major ions occurring in the mass spectra.

Major Findings:

Method Development

The initial phase of this work required the development of a methodology to assay desaturase activity both in *in vivo* and *in vitro* systems using stable isotopically labeled fatty acids by selected ion monitoring (SIM) negative chemical ionization (NCI) GC/MS. During this reporting period, the development of the NCI GC/MS method has been completed. The deuterated materials have been characterized as their pentafluorobenzyl (PFB) and methyl esters by separations on polar FFAP columns and analyzed in the electron impact and also negative and positive ionization modes by mass spectrometry. We have analyzed each of the following fatty acids as PFB esters in the NCI mode: ^{2}H4-18:2w6, ^{2}H4-18:3w6, ^{2}H4- 20:2w6, ^{2}H4-20:3w6, ^{2}H4-20:4w6, ^{2}H5-18:3w3, ^{2}H5-18:4w3, ^{2}H5-20:3w3, ^{2}H5-20:4w3, ^{2}H5-20:5w3, ^{2}H5-22:5w3 and ^{2}H5-22:6w3. In addition, we have increased detection limits by greater than 3 orders of magnitude over the more frequently used positive chemical ionization (PCI) method. With these changes we can now detect and quantify deuterated fatty acids in the low femtogram to high attogram range. Methods for producing the PFB derivatives from polar and neutral phospholipids with subsequent clean-up procedures and recovery analyses have been improved.

Rat Liver In Vitro D6 Desaturase Assay

An *in vitro* Delta-6 desaturase study of rat liver microsomal preparations from animals that had been on diets where the primary source of lipid was corn or fish oil. Half of the animals were exposed to ethanol for a period of seven days. We found that dietary lipids had a marked effect upon liver fatty acid composition and desaturation. Animals fed a corn oil based diet (52% 18:2w6) had an overall higher level of 18:2w6 in liver tissue than those on the fish oil diet (1.5% 18:2w6). The same animals also had higher levels of 20:4w6 in the liver than the fish oil group. Desaturation of ^{2}H4-18:2w6 was lowest in the corn oil group and highest in the fish oil group. In both cases, desaturation was inhibited by ethanol. This data suggests that the desaturase may be regulated by end-product inhibition, however, an alternative explanation would be substrate inhibition.

Ethanol increased the liver concentration of 18:2w6. Although the mechanism for this is not known, it may be due to cofactor competition by alcohol dehydrogenases (ADH) and the desaturase. It is apparent from this data that alcohol plays some role in essential fatty acid metabolism whereby it leads to elevated levels of the fatty acid precursor. These elevated levels were negatively correlated with desaturase activity.

In Vivo Desaturation in Juvenile Rats

The purpose of these experiments was to investigate the brain and liver uptake and conversion of ^{2}H4-18:2w6 and ^{2}H5-18:3w3 to ^{2}H4-20: 4w6 and ^{2}H5-22:6w3, respectively, during a brain growth period. Four day old rat pups were given 1 mg each of the ^{2}H4-18:2w6 and the ^{2}H5-18:3w3 as their ethyl esters by gavage. At 4, 8, 12, 18, 24, 48, 72, 120, and 168 hours the blood,,liver, brain and stomach contents were analyzed for deuterated fatty acids by the methods described above. Results from these experiments have shown that 1) the brain uptake of the ^{2}H4-18:2w6 was between 5 to 10 times that of the ^{2}H5-18:3w3, 2) The conversion of ^{2}H4-18:2w6 to ^{2}H4-20:4w6 was greater than the production of ^{2}H5-22:6w3 from ^{2}H5-18:3w3 during the early time periods in the brain, 3) Production of ^{2}H5- 22:6w3 continued to increase up to 168 hours whereas ^{2}H4-20:4w6 was no longer increasing after 72 hrs, 4) ^{2}H5-22:6w3 appeared in the brain during early time intervals and this may represent uptake from the circulation, 5) Intermediates in the fatty acid synthesis were detected in the brain and liver at the 18 hr interval, 6) The precursors, ^{2}H4-18:2w6 and ^{2}H5-18:3w3 were found in the perfused brain tissue, 7) Liver transformation of ^{2}H4-18:2w6 and ^{2}H5-18:3w3 was greatest at 48 hrs, 8) Release of ^{2}H5-22:6w3 into the blood appeared to be bi-phasic with the greatest rise in ^{2}H5-22:6w3 occurring at 72 hrs.

Preliminary experiments in cats and rhesus monkeys indicate that they are capable of elongation and desaturation although this occurs at a very low rate in the cat. These experiments also indicate the utility of the method for large animals.

Significance to Biomedical Research and to the Program of the Institute:

The development of a non-invasive and highly sensitive method for determination of essential fatty acid elongation and desaturation based on stable isotope technology allows the study of these processes in higher mammals and in humans. There is an extreme paucity of information concerning fatty acid metabolism in animals higher than rats. Animal experiments may be used to determine the relative contributions to polyunsaturated lipid deficiency of the amounts and types of fatty acids in the diet and of alcohol. The mechanisms involved in this altered metabolism appear complex but this assay will provide an important insight into alcohol effects on the desaturase complex of enzymes. This is believed to be one of the primary means involved in the disruption of essential fatty acid levels in the liver, blood stream and brain. As a long term goal, the debate as to the extent to which humans desaturate 18-carbon essential fatty acids as opposed to simply incorporating the preformed long chain metabolites from dietary sources can be settled. It will also make possible a direct evaluation of the metabolic capacity of alcoholics in this regard.

Proposed Course:

We are continuing *in vivo* and *in vitro* studies in rats using the experimental variables of diet, age and alcohol and the end-points of a series of fatty acid elongation and desaturation steps for both omega-3 and omega-6 substrates. We will also begin to assess essential fatty acid metabolism in several higher species. We have initiated a dietary study in domestic cats for the purpose of determining the products of desaturation and chain elongation in a species that is purported to lack delta-6 desaturase activity. We are carrying out an *in vivo* study in Rhesus monkeys due to their phylogenetic relationship to humans and their predisposition to consume alcohol. We will study the *in vivo* metabolism of polyunsaturated fatty acids in this species and determine the effects of alcohol and, in particular, of binge drinking on the enzyme systems.

A clinical study will be designed and implemented that will attempt to measure fatty acid elongation and desaturation in humans. Dietary effects will be evaluated including a low polyunsaturate/ saturate ratio such as may be eaten by an alcoholic and also acute alcohol intake in normal volunteers or in heavy social drinkers. The effect of chronic alcohol abuse on desaturation will be evaluated in alcoholics at the beginning of withdrawal.

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Richard L. Veech, M.D., D.Phil., Chief

Introduction

The three sections of the Laboratory of Metabolism and Molecular Biology (LMMB) are conducting research in seven major areas:

A. The effects of ethanol and its metabolites upon the interlinked system of inorganic ion gradients existing between the intracellular and extracellular water phases exhibiting variably different potentials depending upon the cell type. The regulation of this interlinked system of inorganic ion gradients between phases of differing potential is a fundamental property of living tissue and the ability of ethanol and its metabolites to alter the extent and direction of these gradients has wide ranging effects in: 1) understanding the acute effects of ethanol, 2) the propensity to habituation, and 3) the pathological consequences of chronic ethanol ingestion. These investigations have been carried out on heart, liver, brain and lymphoid tissue *in vivo,* in perfused organs and in cultured cells (Masuda T et al. JBC 1990;265:20321-34). A variety of techniques have been developed in this project including new methods for study of intracellular pH (Dobson GP et al. Anal Biochem 1991;195:232-7) and comparisons of widely used ^{31}P NMR techniques with those of enzymatic analysis. A striking result of these studies is the finding of a decrease of intracellular magnesium ion resulting from alcohol ingestion. A decrease in intracellular magnesium would have widespread metabolic effects in all tissues; but particularly in brain, where a Mg deficiency has been implicated as an etiological factor in severe withdrawal, and in the vascular smooth muscle cells where Mg deficiency has been implicated as a causative factor in hypertension, a common sequelae of heavy alcohol use.

B. A second major area undertaken by the laboratory addressed the effects of ethanol and its metabolites upon the thermodynamics of the metabolites and the kinetics of the enzymes of the *de novo* purine synthesis and purine salvage. The relationship of drinking to gout has been known since classical times, but more recently interest in this area has revived due to the report that ethanol inhibits adenosine reuptake in cultured cells (Nagy LE et al. J Biol Chem 1990;265:1946-51). It has been postulated that abnormalities of adenosine uptake play a role in both the genetic predisposition to develop alcoholism and in abnormalities of signal transduction which are dependent upon cyclic AMP dependent mechanism (Diamond I et al. Proc Natl Acad Sci (USA) 1987;84:1413-6). Fundamental work done in this area will allow investigation of the effects of purine bases such as adenosine to modulate neurotransmitter release, regulate immune function and alter both vascular tone and platelet coagulability.

C. A third major area of research has been an investigation of the effects of ethanol and its metabolites on the alteration of immune function in the alcoholic. This area of research is particularly pertinent during a time when the spread of HIV infection is increased in the addicted segment of the population. Patients undergoing withdrawal from alcoholism were shown to have increased levels of plasma interferon γ(IFNγ) with a resultant increase in cell surface expression of the class I major histocompatibility antigens on circulating leukocytes but a paradoxical decrease in the expression of HLA class II (Jones J et al. Alcoholism, Clin Exptl Studies; in press). It was also shown for the first time, that acetate, the primary metabolite of ethanol, causes an acute elevation of transforming growth factor β (TGFβ) (Anderson J et al. Kidney Intl; in press). TGFβ is a pluripotential cytokine with ability to diminish production of the acute phase response but more importantly to promote the fibrotic response. Understanding the mechanism of hepatic fibrosis and developing methods to treat this process is a major goal of this program since alcoholism is a major cause of cirrhosis, the seventh leading cause of death in the United States, and a significant cause of expensive chronic disability.

D. A fourth major area of work in this laboratory involves determining the control of the gene expression of the cytochrome P450IIE, the major enzyme induced in chronic alcohol abuse. After successfully cloning this gene for the first time, members of the laboratory have now shown that the gene product is expressed in circulating lymphocytes in type I diabetic children (Song BJ et al. J Clin Endocrinol Metab 1990;71:1036-40). The expression of this enzyme in circulating lymphocytes is now being investigated as an objective laboratory test for the laboratory diagnosis of occult alcoholism. Additionally, the post-translational mechanism of P450IIE reduction in the peroxidative CCl_4 toxicity is being investigated.

E. A sixth major area of work in this laboratory is the cloning and expression of the pyruvate dehydrogenase multi-enzyme complex (PDH) (Huh TL et al. J Biol Chem 1990;265:13320-6). PDH is a major control site for the control of the supply of energy for brain and germ-line tissue, which unlike most other tissues, require PDH to sustain life. Isoforms of PDH $E_{1\alpha}$ have been isolated from testis and are being actively pursued in brain, giving for the first time, a possible molecular explanation for the absolute dependence upon glucose metabolism in these tissues. Additionally, the PDH kinase has now been cloned and a new form of protein phosphatase with ability to dephosphorylate $PDHE_{1\alpha}$ has now been identified for the first time. The manner in which ethanol alters the control of the rate of the PDH reaction is being investigated.

F. A seventh major area of work in this laboratory involves use of NMR spectroscopy to develop new methods for the non-invasive measurement of cerebral function. These include the development of a ^{19}F NMR method for estimation of cerebral blood flow (van Zilj PCM et al. Magn Reson Med 1990;16:489-95). Additionally, new methods to non-invasively measure cerebral oxygen consumption using ^{17}O magnetic resonance and intracerebral sodium concentration using double quantum NMR have been undertaken. These new techniques offer the possibility of ultimately being adapted to the study of human alcoholic and other patients.

The work of this laboratory has resulted in the publication of 17 articles during FY'91.

I. The Section on Metabolic Control

This section has continued to make significant progress over the past year with the acceptance for publication of ten papers. Additionally, the Section Chief was an invited speaker during this year at: the Annual Meeting of the Society for

Magnetic Resonance; the Department of Biochemistry, Southwestern University School of Medicine, Dallas, Texas; the Department of Physiological Chemistry, Ohio State University School of Medicine, Columbus, Ohio; the Department of Pharmacology, University of Wisconsin School of Medicine, Madison, Wisconsin; and the Department of Medicine, University of Oklahoma School of Medicine, Oklahoma City, Oklahoma.

A. Alterations in inorganic ion homeostasis in alcoholism:

Recently there has been considerable interest generated on the effects of ethanol upon intracellular calcium. It was reported (Lovinger DM et al. Science 1989;243:1721-4) that 5-50 mM ethanol inhibited the ability of glutamate to allow entry of Ca^{2+} from extracellular space through the NMDA receptor in nervous tissue. In contrast in liver, high doses of ethanol ranging from 300 mM to 1 M have been reported to increase hepatic free [Ca^{2+}] due to stimulation of phospholipase C, elevation of inositol 1,4,5 triphosphate and release of calcium from endoplasmic reticular stores (Hoek JB et al. J Biol Chem 1987;262:682-91). Elevation of extracellular free [Mg^{2+}] blocks the NMDA gated cation channel, and alteration of intracellular free [Mg^{2+}] effects the activity phospholipase C and inositol phosphate kinase. Additionally there is a large body of evidence (Knochel JP. Ann Intern Med 1983;98:849-54) suggesting that calcium accumulation in vascular smooth muscle is a significant cause of the increased incidence of hypertension and cerebrovascular accidents in this population. Alcoholism is significantly associated with hypertension, a major cause of morbidity and mortality in the United States. Recent evidence from *in vivo* studies in animals suggests that acute doses of ethanol result in loss of magnesium for liver and a reciprocal increase in hepatic calcium. The mechanism underlying magnesium movement and the consequences of alteration of cellular calcium homeostasis as well as the metabolic consequences of alteration in free [Mg^{2+}] are currently under study. In particular, the relation of the fundamental energy obtained from the hydrolysis of ATP to the extent and direction of inorganic ion gradients between the intra and extracellular phase is being examined. While the pathological effects of chronic ethanol ingestion upon liver has long been recognized, recent reports (Urbano-Marquez A et al. N Engl J Med 1989;320:409-15) show that about 45% of human subjects who consume 120 g of ethanol per day (the equivalent of 8 drinks) over 20 years have biopsy provable proximal muscle myopathy and also cardiomyopathy. It would thus appear that chronic heavy alcohol use, in addition to producing the well known liver toxicity, can also produce significant myopathy as well.

B. Alterations in purine metabolism: a postulated cause or effect of chronic alcoholism.

Ethanol and its metabolite acetate are known to have effects upon purine metabolism. Ethanol exacerbates clinical gout by diminishing the renal excretion of uric acid and by increasing the rate of purine synthesis. Acetate, the metabolite of ethanol, has been associated with multiple cardiovascular effects, in part, thought to be due to increased adenosine release. More recently, it has been reported that increased extracellular adenosine resulting from chronic ethanol exposure results in inhibition of adenosine uptake and a decrease in the coupling of G-proteins to adenyl cyclase with possible impairment of signal transduction in numerous tissues (Nagy LE et al. J Biol Chem 1991;265:1946-51). Supportive of this hypothesis was the subsequent report that cultured lymphocytes from 76% of patients with a diagnosis of alcoholism had lowered levels of c-AMP (Diamond I et al. Proc Natl Acad Sci 1987;84:1413-6). In view of this evidence, the effects of ethanol and its metabolites on the interrelations of purine metabolism have been examined. The kinetics of the relevant major enzymes of purine metabolism have been determined and the thermodynamics of the reactions assessed both *in vitro* and *in vivo* in rat liver. To my knowledge, this study is the most comprehensive study of the kinetics and thermodynamics of the basic metabolism of purines to have ever been undertaken in one living tissue. It can serve as a basis for evaluating the postulated role of adenosine in producing toxicity of chronic ethanol, as well as the hypothesis that abnormalities of purine metabolism may underlie the predisposition to develop alcoholism.

C. Ethanol and its metabolites as pathogenic agents acting to induce fatal alcoholic hepatitis and hepatic cirrhosis through modifications of the immune system.

The excessive production of NADH which occurs during the metabolism of alcohol in liver has been recognized for many years as an adequate explanation for the pathogenisis of alcoholic fatty liver. However, alcoholic fatty liver is generally a benign condition which reverses itself after cessation of drinking. There is general agreement however, that the precise pathophysiological mechanisms leading to alcoholic hepatitis and alcoholic cirrhosis are not known. Absent such knowledge, the treatments of these conditions remain unsatisfactory. Several recent developments however, offer that this fundamental ignorance is about to change. This recent evidence suggests that a fundamental alteration in the immune system may be playing a role in the pathogenisis of alcoholic liver disease.

Four important developments have occurred within the past two years which point to involvement in the immune system in the pathogenisis of alcoholic hepatitis and cirrhosis:

1) In regard to alcoholic hepatitis, a relatively rare but highly fatal condition with a very high incidence within minority communities, it was recently found that the death of these patients correlated with no known liver function test, but did positively correlate with the presence of detectable tumor necrosis factor α(TNFα) in the plasma (Felver ME et al. Alcoholism, Clin Exptl Res 1990;14:255-9). TNFα is a powerful cytokine produced by cells of the monocytic series and capable of inducing cell death.

2) Chronic ethanol treatment of rats results in an increase of hepatic natural killer cell activity.

3) Earlier work at another NIH Institute had shown that alcoholics have increased levels of HLA class I antigen present on their circulating mononuclear cells (Parent LJ et al. Faseb J 1987;1:469-73). This year the laboratory showed that withdrawal of human alcoholics is associated with increased plasma levels of interferon γ(IFNγ) (Jones J et al. Alcoholism, Clin Exptl Stud; in press). The increase in IFNγ during withdrawal from alcohol raises the possibility that IFNγ levels are actively suppressed by alcohol. The observation that IFNγ is increased during withdrawal offers an explanation of the observation of an increase in HLA class I surface antigen in mononuclear cells from alcoholics, since IFNγ, is the most powerful known stimulant for the expression of these

surface active molecules associated with the activation of cells of the monocyte and macrophage class.

4) The exposure of patients to the ethanol metabolite, acetate results within 4 hours in an increase in the plasma levels of transforming growth factor β(TGFβ), a powerful cytokine known to stimulate Kupffer cells to produce collagen and fibroblasts to synthesize extracellular matrix. The evidence from a number of different studies suggesting multiple alterations in immune function in the alcoholic thus provide, for the first time, the beginning of an understanding of these serious and often fatal medical consequences of alcohol abuse. Furthermore, the activation of monocytes resulting from IFNγ production during alcohol withdrawal, has important implications for increasing the activation of the C4 helper T cell activation within the alcoholic population, which is at high risk for infection with HIV-1 infection. These advances in our understanding that a small molecule like ethanol can alter immune system function offers promise for improved therapy in major causes of morbidity within the U.S.

The work in these areas resulted in this Section publishing ten (10) papers in FY'91.

II. The Section On Molecular Genetics

The Section on Molecular Genetics has achieved two goals this year:

A. The characterization of a new class of protein kinase and a new protein phosphatase responsible for the phosphorylation and dephosphorylation of $E_1\alpha$ subunit of the intramitochondrial pyruvate dehydrogenase complex.

B. The study on the additional mechanisms of regulation of cytochrome P450IIE, ethanol-inducible cytochrome P450.

A. The Characterization and Molecular Cloning of the Subunits of the Pyruvate Dehydrogenase Multienzyme Complex:

The major effort of this Section has been directed toward the characterization and molecular cloning of the subunits of the pyruvate dehydrogenase (PDH) multienzyme complex. The PDH multienzyme complex is composed of at least 5 different subunits: E_1 (pyruvate dehydrogenase), E_2 (lipoyl acetyltransferase), E_3 (lipoamide dehydrogenase) and two regulating enzymes of PDH-specific kinase and PDH-specific phosphatase. The PDH complex is known to be regulated not only by various allosteric regulators but also by a reversible cycle of protein phosphorylation and dephosphorylation catalyzed by specific PDH-kinase and PDH-phosphatase, respectively. This Section had previously cloned three subunits of the PDH complex and characterized the regulatory mechanisms of PDH gene expression in cultured fibroblasts obtained from patients with varying degrees of lactic acidosis. After purifying the PDH complex and the PDH-specific kinase (M_r 48,000 and 45,000 on SDS-polyacrylamide gel) and phosphor-PDH-phosphatase (M_r 50,000 and 90,000 on SDS-polyacrylamide gel) to near homogeneity, they have successfully determined the partial amino acid sequences of these proteins whose structures have not been characterized. Based on the amino acid sequences, oligodeoxynucleotides were synthesized and used for cloning the cDNAs for both regulating enzymes. Several oligopeptides were also synthesized and used for the generation of polyclonal antibodies against these subunits. By using these oligonucleotides and anti-peptide antibodies, they have now achieved the first cloning of the PDH-kinase. The PDH-kinase represents a new class of serine protein kinase distinct from other classes of protein kinases dependent upon cAMP, cGMP, or Ca^{2+}-calmodulin. Many investigators in the field have attempted to clone cDNAs for this protein kinase but failed.

Several cDNA clones coding for the regular heterodimeric PDH-phosphatase were also isolated and being characterized by DNA sequencing to match the partial amino acid sequences. This PDH-phosphatase is also unique in substrate specificity and dependence on other cellular regulators. The cloning and study on the expression of the two regulating enzymes should add to our fundamental knowledge of the control of a central enzyme in the metabolism of both carbohydrates and amino acids.

A new protein phosphatase against phosphorylated PDH complex was purified to homogeneity from bovine heart. The amino acid sequencing of the several tryptic peptides of this monomeric PDH-phosphatase (M_r 68,000 on SDS-polyacrylamide gel) confirmed that it is identical with a major Ca^{2+} binding protein, p68 or calelectrin, whose catalytic function has not been reported. The precise subcellular localization and its role in dephosphorylating or reactivation of the PDH complex as compared to the regular heterodimeric PDH-phosphatase are being investigated.

In addition to the general importance of the PDH complex, it is of specific interest in the field of alcoholism because of the occurrence of elevated levels of abnormal metabolites, *meso*- and *d*- or *l*-2,3-butanediol found in the blood of human alcoholics. During this year, a pathway leading to the production of the *meso*-form of this metabolite has been postulated. This was evidenced in rats by treatment with short chain fatty acids indicating an involvement of the PDH multienzyme complex. The *meso*-2,3-butanediol, in addition to being found in elevated levels in the blood of alcoholics, is also found in excessive amounts in non-alcoholic patients with hepatic cirrhosis and with the genetic disease of methyl melanuria. The identification of the pathway of *meso*-2,3-butanediol adds interest to the observation that the optically active form of the compound, either *d* or *l*, which now appears to be specific to either alcoholics when drinking, or alcoholic cirrhosis in the absence of drinking. Based on our observation, these fundamental studies of the metabolic pathways, and the enzyme responsible for their formation, would ultimately add to our understanding either of the etiology or the pathophysiology of human alcoholism.

B. Study on the Regulatory Mechanisms of Cytochrome P450IIE Gene Expression.

The chronic drinking of ethanol causes an induction of cytochrome P450IIE (ethanol-inducible P450IIE). This protein is associated with the microsomal fraction and responsible for the metabolism of various substrates including ethanol, other clinically used drugs, and water soluble carcinogens. The cDNA and gene for this protein was first cloned by members of this Laboratory and subsequently characterized extensively for the multiple mechanisms of its regulation which contrast other classes of cytochromes P450s. They have also demonstrated that this protein is present in lymphocytes and induced in certain conditions such as diabetes and starvation. Its level positively correlated with the level of hemoglobin A_{1c} which is widely used as a metabolic control of these individuals. In other studies, we also demonstrated that another abnormal metabolite, 1,2-propanediol, can result from the conversion of ketone bodies, through acetone and acetol to 1,2-propanediol, and ultimately to glucose through the action of this enzyme.

During this year, we have demonstrated that two ketone bodies (acetoacetate and β-hydroxybutyrate) may be responsible for the pre-translational P450IIE induction observed in ketotic condition caused by a high fat diet as similar to those observed in starved or diabetic animals. In addition, the post-translational reduction of P450IIE by its substrate, CCl_4 was clearly demonstrated. The mechanism of specific decrease in the P450IIE during pregnancy was being studied and its putative regulators are being investigated. The additional mechanisms of P450IIE regulation should add to our fundamental knowledge of the multiple regulation of P450IIE which is quite different from other classes of cytochromes P450, most of which are transcriptionally activated.

Because of its potential roles in drug-related toxicity and in human carcinogenesis of esophageal cancer, P450IIE has been well studied. Having recognized P450IIE expression and induction in lymphocytes from human and experimental animals, we are actively determining its levels in lymphocytes from human alcoholics as an aid in the objective diagnosis of excessive alcohol consumption or potentially other pathological conditions. Early diagnosis of excessive drinking would help to treat these patients successfully and prevent other undesirable conditions or morbidity associated with alcoholism.

III. Section on Physical Chemistry

The work of the Physical Chemistry Section is divided into four areas:

(A) Development of new non-invasive NMR methods for measurement of blood flow, metabolic rate, metabolite levels, and transmembrane ion gradients in animals and humans.

(B) Development of new in vivo fluorescence techniques for determination of intracellular calcium levels in the intact brain.

(C) Investigation of the role of the hypothalamus/hypophysis/adrenal gland axis and the sympathetic nervous system in the control of cerebral blood and metabolic rate in different models of stress.

(D) Investigation of effects of acute and chronic exposure to ethanol, and withdrawal from chronic alcohol consumption, on cerebral blood flow and oxygen consumption.

(A) In the last year the Physical Chemistry Section developed a new 17O NMR approach for imaging oxygen consumption in the brain. The Section also continued work on two previous projects - the use of multiple quantum 23Na NMR techniques to measure intracellular sodium levels in intact organs, and the use of 19F NMR to measure cerebral blood flow. In the last year the hypothesis that multiple quantum 23Na NMR selectively detects intracellular sodium ions has been tested and confirmed in two ways, and multiple quantum 23Na techniques have been combined with 31P NMR techniques to follow cellular energetics and intracellular sodium levels simultaneously. Also, in the last year the 19F NMR approach for measuring cerebral blood flow, using CHF_3 as an inert tracer, has been extended to image cerebral blood flow with 0.8 cc voxel size.

One of the long-term goals of the ^{17}O, ^{23}Na and ^{19}F NMR projects is to develop new non-invasive approaches that can be used to measure organ function in the clinical studies.

In the last year, the Physical Chemistry Section started a new project on the use of non-invasive ^{31}P NMR techniques to study metabolic alterations in skeletal muscle during exercise protocols. In the coming year this approach will be used to study panic disorder patients and chronic alcoholic patients.

(B) In the last two years the Physical Chemistry Section modified a fluorescence approach that has been used to measure intracellular calcium levels in isolated cells, so that the approach can be used in the intact brain. This approach has been combined with ion-sensitive electrode techniques to give a simultaneous measure of intracellular calcium, extracellular calcium and extracellular sodium levels in the intact brain. The long-term goal of this project is to study the effects of alcohol on transmembrane calcium gradients in the brain.

(C) In the last three years the Physical Chemistry Section investigated the role of the hypothalamus/hypophysis/adrenal gland axis and the sympathetic nervous system on the control of cerebral blood flow and metabolic rate in different model of stress. Most of the work has utilized hypercapnia as a simple model for stress. Work in the first two years of the project established that, in response to hypercapnia, the adrenal medulla secretes an unknown substance that activates cerebral blood flow and metabolism. Work in the last year strongly suggests that this substance is a μ-opiod peptide. Also, work in the last year strongly implicates the sympathetic nervous system in the response of cerebral blood flow and metabolism of hypercapnia. Finally, in the last year the general approach has been extended to studies with another model for stress-hypoglycemia.

The long-term goal is to apply the techniques developed in this project to study the role of the hypothalamus/hypophysis/adrenal gland axis and the sympathetic nervous system on the response of the brain to acute alcohol intoxication and withdrawal from chronic alcoholic intoxication.

(D) In the last two years the Physical Chemistry Section studied the effects of acute alcohol intoxication on cerebral blood flow and oxygen consumption. These studies utilized a modification of the Kety-Schmidt approach that could be used with conscious, freely-moving rats. These studies are currently being extended to include the effects of chronic alcohol intoxication and withdrawal from chronic alcohol intoxication on cerebral blood flow and oxygen consumption.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00044-02 LMMB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Use of Site Directed Mutagenesis to Study the Mechanism of Action of Pig Citrate Synthase

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	J.P. Casazza	Research Chemist	LMMB, NIAAA
Others:	None		

COOPERATING UNITS *(if any)*
Basic Biochemistry Department, VA Medical Center, Dallas (P. Srere, C. Evans) and Department of Biological Chemistry, Wayne State University School of Medicine (G. Alter).

LAB/BRANCH
Laboratory of Metabolism and Molecular Biology

SECTION
Metabolic Control

INSTITUTE AND LOCATION
NIAAA, 12501 Washington Avenue, Rockville, Maryland 20852

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.3	0.3	

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Prior to the development of molecular biology physicochemical studies of enzymes were limited to studying native enzymes, proteolysed enzymes and in certain cases chemically modified enzymes. With the advent of site directed mutagenesis and bacterial expression vectors with strong promoters, it has become possible to ask questions about the role of specific amino acids in catalysis, selectively change these amino acids, produce large amounts of the mutant protein in culture, and then study the physicochemical properties of the purified protein. We have modified His274 and Asp375 in pig citrate synthase, two amino acids identified as being at the active site of the enzyme by X-ray crystallogrophy. These amino acids were replaced not only with uncharged amino acids, but also with amino acids with different pKa's, charge and chain lengths. Kinetic analysis of these mutant enzymes along with already published crystallographic data suggest that, (1) prior to the actual condensation reaction His274 and Asp375 act in concert, maintaining the electrostatic neutrality of the His-Asp pair while facilitating the enolization of acetyl CoA without the formation of the enolate ion and (2) during the actual condensation reaction His274 accepts a proton from the hydroxide of the enol intermediate as ASP375 protonates the reaction His274 accepts a proton from the hydroxide of the enol intermediate as Asp375 protonates the thioester sulfer, forming the sulfonium ion, thus facilitating the displacement of coenzyme A by oxaloacetate.

THIS PROJECT HAS BEEN TERMINATED.

PHS 6040 (Rev. 1/84) GPO 914-918

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00045-02 LMMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

In Vivo Portal-Hepatic Venous Gradients of Glycogenic Precursors in the Rat

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	G. P. Dobson	Visiting Associate	LMMB, NIAAA
Others:	R. L. Veech	Chief	LMMB, NIAAA
	J. V. Passonneau	Research Chemist	LMMB, NIAAA

COOPERATING UNITS *(if any)*

Department of Biochemistry, Chang Gung Medical College, Tao-Yuan, Taiwan R.O.C. (M.T. Huang)

LAB/BRANCH

Laboratory of Metabolism and Molecular Biology

SECTION

Metabolic Control

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Rockville, Maryland 20852

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
.6	.6	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Following the pioneering experiments in the mid-nineteenth century of Claude Bernard, it was fairly well established that dietary glucose in the post-prandial state was taken up directly by the liver and incorporated into newly synthesized glycogen. About 10 years ago this viewpoint was strongly contested by the radiotracer studies of Newgard et al., (JBC 1984;258:8046-52), later to be supported by Drs. J. Katz and B. Landau. Their position was that the metabolic fate of dietary glucose was not the direct incorporation into liver glycogen, but rather it was first broken down to C3 units by unspecified organs of the body which then returned to the liver to form glycogen via gluconeogenesis. This latter pathway was called the "Indirect Pathway", and an entirely new field of inquiry termed "The Glucose Paradox" was born. We challenged this interpretation and carried out experiments on chronically cannulated male Wistar rats which were fed ground chow for 2 hr for six days. On the 7th post-operative day, blood was simultaneously drawn from the portal and hepatic veins over a 2 hr feeding period. The position of the hepatic vein cannula was verified using a tritiated water washout technique. In separate experiments, 200 uCi of [3-^{3}H]glucose was added to the food in order to determine the relative contribution of D-glucose and 3-C precursors to newly synthesized glycogen. Our results demonstrate that 73% of newly synthesized liver glycogen formed during the 2 hr of feeding came directly from the uptake and phosphorylation of dietary glucose from the gut via the portal vein without prior conversion to 3-C precursors.

THIS PROJECT HAS BEEN TERMINATED

Dobson GP, Veech RL, Passonneau JV, Huang M-T. In vivo portal-hepatic venous gradients of glycogen precursors and incorporation of [3-^{3}H]-D-glucose into liver glycogen in the awake rat, J Biol Chem 1990;265:16350-7.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 AA 00046-02 LMMB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Quantitation of 31P NMR

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboretory, and institute affiliation)*

P.I.:	G.P. Dobson	Visiting Associate	LMMB, NIAAA
Others:	J.V. Passonneau	Research Chemist	LMMB, NIAAA
	R.L. Veech	Chief	LMMB, NIAAA

COOPERATING UNITS *(if eny)*
Dept. Biochem. Biophys., University of Pennsylvania, PA (K. Kobayashi, T. Inubushi, S. Wehrli, S. Nioka, B. Chance)

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1	1	

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☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use stenderd unreduced type. Do not exceed the spece provided.)*

Like any spectroscopic method, the usefulness of ^{31}P NMR as a quantitative analytical technique depends upon establishing whether the areas under the phosphorus peaks are proportional to the relative number of ^{31}P nuclei in the phosphorus compound in the sample. The aim of this study was to compare the ^{31}P NMR estimates of PCr, Pi and pH in the isolated rat heart with those determined from enzymatic procedures. The major findings of the study were (1) ATP and PCr are equally visible in the isolated working rat heart, (2) the NMR estimate of intracellular pH and enzymatic tissue bicarbonate method (Dobson et al., Anal Biochem, in press.) agree to within 0.1 pH unit, and (3) with the exception of glucose-perfused hearts at high work, the cytosolic Pi content calculated by either the NMR or the near-equilibrium method was 30 to 70% of the total tissue Pi content indicating that a considerable fraction of the total was compartmented, presumably in the mitochondria.

This project has been terminated.

Project Description:

Investigators:

G.P. Dobson	Visiting Associate	LMMB, NIAAA
B. Chance	Professor Emeritus	Dept. Biochem. Biophysics Univ. Pennsylvania, PA
T. Inubushi	Research Scientist	Dept. Biochem. Biophysics Univ. Pennsylvania, PA
K. Kobayashi	Research Scientist	Dept. Biochem. Biophysics Univ. Pennsylvania, PA
S. Nioka	Research Scientist	Dept. Biochem. Biophysics Univ. Pennsylvania, PA
J.V. Passonneau	Research Chemist	LMMB, NIAAA
R.L. Veech	Chief	LMMB, NIAAA
S. Wehrli	Research Scientist	Dept. Biochem. Biophysics Univ. Pennsylvania, PA

Objectives:

The aim of this study was to compare the ^{31}P NMR estimates of PCr, P_i and pH in the isolated rat heart with those determined from enzymatic procedures. With respect to P_i, the NMR method which detects predominantly cytosolic P_i, and this value will be compared to the cytosolic P_i calculated from near-equilibrium approach using the tissue components of the cytosolic CK, PGK-GAPDH-LDH catalyzed reactions. pH will by NMR also be compared with a bicarbonate developed by GP Dobson at NIAAA.

Methods Employed:

This study was a collaborative effort between the NIAAA and Dr. Chance's laboratory at the University of Pennsylvania. The state of the art NMR 11.8 Tesla spectrometer from Bruker was used. Hearts were perfused by the method of Kobayashi and Neely. Routine enzymatic analysis was performed at the LMMB, NIAAA.

Major Findings:

Despite ^{31}P NMR being a potentially important tool to non-invasively monitor tissue metabolic changes in states of health and disease, no single study has addressed the quantitative aspects of the method by comparing it to conventual chemical methods. Hearts from 200-350 g fed male Wistar rats were perfused at low and high workloads with P_i-free Krebs-Henseleit medium containing either 10 mM glucose or 10 mM glucose plus 15 mU/ml insulin. The intracellular pH by ^{31}P nmr ranged between 6.99 and 7.02 and agreed to within 0.1 pH unit of estimates calculated using enzymatically determined total tissue HCO_3^-/CO_2 contents. At high work, where the tissue contents of PCr and ATP were determined on the same heart as NMR areas (n=16), the proportionality factors, defined as the ^{31}P NMR area units divided by the total enzymatic tissue content (area units/umol g^{-1} wet wt heart), was 112 ± 8 for PCr; 99 ± 4 for g-ATP; 138 ± 9 for a-ATP and 100 ± 4 for b-ATP. It was concluded that ATP and PCr were equally visible by ^{31}P NMR and that no significant breakdown of PCr from tissue acid extraction occurred. The other part of the study focused on the estimation of cytosolic P_i. The cytosolic P_i estimated from NMR in glucose perfused hearts at low and high work was 0.92 ± 0.11 and 0.92 ± .08 umol/g wet wt. Using the near-equilibrium expression of K_{CK}/K_{G+G} and the metabolic levels in heart extracts, the calculated cytosolic P_i was 1.45 ± 0.19 and 3.26 ± 0.50 umol/g wet wt respectively. The cytosolic NMR P_i in the glucose plus insulin hearts was 0.54 ± 0.07 and 0.76 ± 0.05 umol/g wet wt at low and high work and 1.57 ± 0.22 and 1.94 ± 0.37 umol/g wet wt from near-equilibrium estimates. The total tissue P_i measured enzymatically ranged from 2.16 to 3.17 umol/g wet wt heart. The validity of using both the NMR and near-equilibrium method for estimating sytosolic P_i in the heart was discussed.

Significance to Biomedical Research and the Program of the Institute:

Like any spectroscopic method, the usefulness of ^{31}P NMR as a quantitative analytical technique depends upon establishing whether the areas under the phosphorus peaks are proportional to the relative number of ^{31}P nuclei in the phosphorus compound in the sample, whether it be in a clinical situation or in the laboratory. This study was designed to compare the ^{31}P NMR estimates of PCr, P_i and pH in the isolated rat heart with those determined by conventional enzymatic procedures.

Proposed Course:

The collaborative study between the Laboratory of Metabolism and Molecular Biology, NIAAA and the University of Pennsylvania has been completed and the subject will no longer be continued.

Publications:

Dobson GP, Veech RL, Passonneau JV, Kobayashi K, Inubushi T, Wehrli S, Nioka S, Chance B. ^{31}P NMR and Enzymatic Analysis of Cytosolic PCr, ATP, P_i and Intracellular pH in the Isolated Working Perfused Rat Heart, NMR in Biomedicine 1991, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00054-01 LMMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The Effects of Ethanol on Brain Metabolism

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	G.P. Dobson	Visiting Associate	LMMB, NIAAA
Others:	W.E. Teague	Chemist	LMMB, NIAAA
	R.L. Veech	Chief	LMMB, NIAAA

COOPERATING UNITS *(if any)*

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1	1	

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Intra-peritoneal injections of ethanol (50 umoles/ml body water) Sodium D(-)lactate (25 umoles/ml body water), and pentobarbital (15mg/kg rat) into 48 hr fasted male wistar rats produced anesthesia in about 15 minutes. Compared to controls, rats under ethanol and sodium D(-)lactate and sodium pentobarbital anesthesia showed a significant 40 to 50% decrease in their brain mitochondrial redox potential as assessed from a change in the $NAD^+/NADH$ ratio. The cytosolic redox potential remained unchanged with a lactate/pyruvate ration ranging from 15 to 23. The cytosolic phosphorylation potential was not significantly different between the ethanol group, the D(-) and L(+) lactate groups, pentobarbital group and the controls.

On the basis of a decreased mitochondrial NAD/NADH ration, the current working hypothesis is that ethanol, D-lactate and Na-pentobarbital all exert a common effect to induce anesthesia by causing a partially inhibition of electron transport. This suggestion would help explain why tissues like brain and heart accumulate NADH, even though in contrast to liver, they lack alcohol dehydrogenase.

Project Description:

Investigators:

G.P. Dobson	Visiting Associate	LMMB, NIAAA
W.E. Teague	Chemist	LMMB, NIAAA
R.L. Veech	Chief	LMMB, NIAAA

Objectives:

To investigate the effect of anesthetic doses of ethanol, D-lactate and pentobarbital on brain metabolism, free magnesium and ion gradients in the rat.

Methods Employed:

Rats were injected intra-peritoneally. After 20-30 minutes brain tissue was obtained at lipid nitrogen temperatures using the brain blower. Ions were measured using a Perkin-Elmer atomic absorption spectrophotometer. Heart metabolites were measured using routine fluorometric or spectrophotometric enzymatic coupled reaction procedures.

Major Findings:

Intra-peritoneal injections of ethanol (50 umoles/ml body water) Sodium D(-)lactate (25 umoles/ml body water) into 48 hr fasted male wistar rats weighing 160-225g produced a coma in about 15 minutes. No apparent anesthesia type effect was observed in control rats receiving identical concentrations of sodium L(+)-lactate, or in those rats receiving bolus injections of 20 and 2.5 umoles/ml body water of sodium chloride. After 30 minutes, the brain was removed and quick frozen at liquid nitrogen temperatures using the freeze-blowing technique.

By measuring the components of the glutamate dehydrogenase equilibrium, rats under ethanol and sodium D(-)lactate anesthesia showed a significant 50-40% decrease in their brain mitochondrial NAD$^+$/NADH redox potential compared to saline injected controls of 1.98±.18 (n=6). Similarly, rats injected with the anaesthetic sodium pentobarbital also showed a 40% reduction in the mitochondrial. In contrast to D-lactate, L-lactate had no effect on mitochondrial redox with a NAD$^+$/NADH of 2.06±0.10. The lactate/pyruvate ratio was 15±0.6 in controls, 23±1.2 in ethanol and 15±0.6 in D-lactate injected rats demonstrating that the cytosolic redox potential remains relatively constant. The cytosolic phosphorylation potential was not significantly different between the ethanol group, the d(-) and L(+) lactate groups, pentobarbital group and the controls as judged from the creatine kinase equilibrium with values ranging from 40,000 to 45,000 M^{-1}.

On the basis of a decreased mitochondrial NAD/NADH ratio, the current working hypothesis is that ethanol, D-lactate and Na-pentobarbital all exert a common effect to induce anesthesia by causing a partially inhibition of electron transport. This suggestion would help explain why in tissues like brain and heart accumulate NADH, even though in contrast to liver, they lack alcohol dehydrogenase.

Significance to Biomedical Research and the Program of the Institute:

Understanding the effects of ethanol-induced anesthesia in relation to other anesthetics on brain metabolism is of fundamental importance. The major findings of the present research report that the effect of ethanol causes a mitochondrial reduction is a new and important one and suggests a link between anesthesia, the redox and phosphorylation potentials and ion gradients across the cell and mitochondrial membranes.

Proposed Course:

The proposed course is to quantitate the distribution of ions and the free magnesium across the plasma membrane in the brain of control versus ethanol/D-lactate/pentobarbitol rats. Oxygen consumption of the brain during these different metabolic states may also be measured. It is hoped this proposed course will throw some further light upon the possible mechanisms involved in the nature of anesthesia and its relation with ethanol-induced intoxication.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00055-01 LMMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Effects of Ethanol on Cardiac Performance

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	G.P. Dobson	Visiting Associate	LMMB, NIAAA
Others:	K. Sato	Visiting Fellow	LMMB, NIAAA
	W.E. Teague	Chemist	LMMB, NIAAA
	R.L. Veech	Chief	LMMB, NIAAA

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Metabolism and Molecular Biology

SECTION

Metabolic Control

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Rockville, Maryland 20852

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1	1	

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Hearts excised from 400-500g male wistar rats were perfused in the working mode. The mean aortic pressure was 80 mmHg and the mean left atrial pressure or preload at 10 cm H_2O. The perfusion medium comprised 10 Mm glucose (no insulin), 117 Mm NaCl, 5.9 Mm KCl, 25 Mm $NaHCO_3$, 1.2 Mm NaH_2PO_4, 1.12 Mm $CaCl_2$ (free Ca^{2+}=1.07 Mm), 0.512 Mm $MgCl_2$ (free Mg^{2+}=0.5mM) pH7.4 at 38 degrees C, with and without 75 Mm ethanol. The perfusate was vigorously bubbled with 95% O_2, 5% CO_2, and was not recirculated in the heart. In the presence of 75 Mm ethanol, the cardiac output fell by 40%, aortic flow by 60%, oxygen consumption by 17% and the power output by 42%. Mean values for cardiac output, aortic flow, oxygen consumption and power output in control hearts were 38.6 ml/min, 19.7 ml/min, 9.83 umol O_2/min/g left ventricle and 415 mjoules/min/g left ventricle respectively (n=7). The presence of ethanol in the perfusion medium resulted in a 23% reduction in heart mitochondria (NAD/NADH ratio decreased from 3.95 to 3.04), a change that was similar in magnitude and direction to the decrease in the oxygen consumption (17%).

The most important finding of this study on the acute effects of ethanol on heart function, was that the magnitude of the fall in oxygen consumption in the presence of 75mM ethanol was only 40% of the decrease in power output indicating a mismatch between supply and demand. This result may have potential clinical implications.

Project Description:

Investigators:

G.P. Dobson	Visiting Associate	LMMB, NIAAA
K. Sato	Visiting Fellow	LMMB, NIAAA
W.E. Teague	Chemist	LMMB, NIAAA
R.L. Veech	Chief	LMMB, NIAAA

Objectives:

The aim of the study was to determine the acute effects of ethanol on cardiac work, oxygen consumption and metabolism.

Methods Employed:

Hearts were perfused using the isolated perfused rat heart preparation of Neely and Morgan. Oxygen and CO_2 tension, and pH in the arterial and venous perfusion lines was measured using a CIBA-CORNING 200 Acid-base analyzer. Ions were measured using a Perkin-Elmer atomic absorption spectrophotometer. Heart metabolites were measured using routine fluorometric or spectrophotometric enzymatic coupled reactions.

Major Findings:

Hearts were excised from 400-500g male Wistar rats were perfused in the working mode. The mean aortic pressure was 80 mmhg the mean left atrial pressure or preload at 10 cm H_2O. The perfusion medium comprised 10 Mm glucose (no insulin), 117 Mm NaCl, 5.9 Mm Kcl, 25 Mm $NaHCO_3$, 1.2 Mm NaH_2PO_4, 1.12 Mm $CaCl_2$ (free Ca^{2+}=1.07 Mm), 0.512 Mm $MgCl_2$ (free Mg^{2+}=0.5 Mm) Ph 7.4 at 38 degrees C, with and without 75 Mm ethanol. The perfusate was vigorously bubbled with 95% O_2, 5% CO_2, and was not recirculated in the heart.

Compared to control hearts, 75 Mm ethanol hearts had a small but significant 7% decrease in total intra-cellular magnesium (18.87 to 17.52 Mm intra H_2O), 1 16% increase in Calcium from 0.55 to 0.64 Mm (not significant p<.05), a 10% decrease in chloride from 48.62 to 43.92 Mm (not significant p<.05), a 5% decrease in chloride from 143.5 to 135.9 Mm (not significant p<.05), and a 4 fold increase in intra-cellular sodium from 5 to 20 Mm. The effect of ethanol on extra-cellular space in heart is required before the effect on ethanol on ion distribution can be quantitated. Free magnesium could not be estimated on the basis of the low citrate/isocitrate ratios (1.84 to 3.24 in control and ethanol hearts respectively).

Mean values for cardiac output, aortic flow, oxygen consumption and power output in control hearts were 38.6 ml/min, 19.7 ml/min, 9.83 umol O_2/min/g left ventricle and 415 mjoules/min/g left ventricle respectively (n=7). In the presence of 75 Mm ethanol, the cardiac output fell by 40%, aortic flow by 60%, oxygen consumption by 17% and the power output by 42%. A striking observation was that the magnitude of the fall in oxygen consumption in the presence of 75 Mm ethanol was only 40% of the decrease in power output indicating a mismatch between supply and demand. The presence of ethanol in the perfusion medium resulted in a 23% reduction in heart mitochondria (NAD/NADH ratio decreased from 3.95 to 3.04), a change that was similar in magnitude and direction to the decrease in the oxygen consumption (17%).

Significance to Biomedical Research and the Program of the Institute:

The striking finding of the acute effects of 75 Mm ethanol to cause a 40% reduction in cardiac output and work compared to a 17% fall in oxygen consumption may have important clinical implications. This may be particularly significant during physical exercise after the ingestion of large amounts of ethanol. Although our findings are from an *in vivo* model, a literature survey also revealed a very similar finding about 30 years ago *in vivo* in the intact dog. Furthermore, the acute effects of ethanol on heart metabolism had the same effect to decrease the mitochondrial NAD/NADH ratio as reported in a parallel study on the acute effects of ethanol on brain metabolism (see progress report LMM-MC-1, 1991). The full significance of the ethanol induced change in mitochondrial redox is currently being explored.

Proposed Course:

There are two aspects of this research project that requires further study. First, the quantitation of the acute effects of ethanol on oxygen consumption and myocardial performance over a wide range of physiological levels of ethanol are required. Further, and of possible clinical significance, determine the relationship between oxygen consumption and power output at different levels of cardiac work by raising the afterload. Intra-extracellular space measurements need to be completed in order to quantitate the effect of ethanol on distribution of ions across the plasma membrane of heart.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00047-02 LMMB

PERIOD COVERED

October 1, 1990 to September 30 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Effects of alcohol and acetate on leukocyte membrane receptor expression

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	J.M. Jones	Visiting Scientist	LMMB, NIAAA
Others:	R.L. Veech	Chief	LMMB, NIAAA

COOPERATING UNITS *(if any)*

National Institute of Dental Research (F. Abbasi); Johns Hopkins University, Baltimore, MD (G. Briefel, K. Yu, O. Yeralan, J. Anderson, E. Mezey).

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1	1	

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☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose was to determine whether chronic exposure to moderate to high blood levels of acetate would result in alterations of expression of selected membrane markers. For this purpose, we examined alcoholic subjects exposed to acetate via alcohol metabolism (up to 10 fold physiological levels) and hemodialysis patients exposed via the dialysis buffers (up to 100 fold physiological levels). Acute exposure to high levels of alcohol was reported by others to enhance the expression of HLA class I antigens. In the present study, the expression of HLA class I (ABC), Class II (Dr) and CD16 Fc receptor antigens were measured by flow cytometry on blood leukocytes from alcoholic and hemodialyzed uremic patients. By staining with fluor=labeled monoclonal antibodies, we detected a small but not significant increase in expression (intensity) of HLA class I antigens on lymphocytes and monocytes of alcoholic subjects in comparison with a reference population of normal controls. Hemodialysis subjects exhibited a decreased expression of HLA class I. Alcoholic and hemodialysis subjects both showed a significant decrease in expression of HLA class II antigens. The average percentage of lymphocytes from alcoholics that expressed CD16 was similar to that of controls. Hemodialyzed subjects exhibited a decrease percent of lymphocytes that stained for CD16 but an increase percent of monocytes that stained for CD16. The results were compatible with a hypothesis that altered expression of HLA class I reported by others was an alcohol effect while the altered expression of class II and CD16 that we observed was an effect caused by uremia, but not by acetate.

Project Description:

Investigators:

J.M. Jones	Visiting Scientist	LMMB, NIAAA
R.L. Veech	Chief	LMMB, NIAAA
F. Abbasi	Physician	NIDR
K. Yu	Physician	JHU
O. Yeralan	Physician	JHU
G. Briefel	Chief Nephrologist	JHU, MD
J. Anderson	Physician	JHU, MD
E. Mezey	Professor	JHU, MD

Objectives:

To examine membrane receptors.

Methods Employed:

Flow cytometry after staining with fluorescent-labeled antibody.

Major Findings:

(1) HLA Class I elevation in alcoholics.

(2) HLA Class I and Class II depressed in hemodialysis.

(3) Lymphocyte CD16 decreased in hemodialysis but monocyte CD16 increased.

Significance to Biomedical Research and the Program of the Institute:

Alcohol may enhance expression of HLA class I; and uremia with or without hemodialysis may depress expression of HLA class I and II and CD16 Fc receptors. These events would be predicted to have immunomodulating influences.

Proposed Course:

Examine other leukocyte Fc receptors and evaluate immune cellular functions in dialyzed patients. Explore the genetic and molecular mechanisms responsible for the observed alteration.

Publications:

Jones JM, Veech RL, Abbasi F, Yu K, Yeralan O, Briefel GR, Anderson J, Mezey E. Altered Expression of HLA antigens and CD16 Fc Receptors on Leukocytes of Alcoholic Subjects and Uremic Patients, Alcoholism, Clin Exp Res, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00048-02 LMMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Distribution in the Perfused Rat Hearts: Effect of Pi and Ethanol

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	T. Masuda	Visiting Fellow	LMMB, NIAAA
Others:	G.P. Dobson	Visiting Associate	LMMB, NIAAA
	K. Sato	Visiting Fellow	LMMB, NIAAA
	R.L. Veech	Chief	LMMB, NIAAA

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Metabolism and Molecular Biology

SECTION

Metabolic Control

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Rockville, Maryland 20852

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2	2	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Isolated hearts were perfused in modified Krebs-Henseleit buffer (118 mM NaCl, 5.9 mM KCl, 1.07 mM free Ca2+, 0.5 mM free Mg2+, 25 mM NaHCO3, and 10 mM glucose, pH 7.4 at 38oC) containing 0, 1.2 or 5 mM Pi. The total H20 content was 0.853 ml/g wet wt. of which 59.3% (14C-mannitol space) was extracellular and independent of [Pi]. No significant change in intracellular Pi content was found with values ranging from 15.8 to 20.5 umol/ml cell H20. Increasing Pi from 0 to 5 mM significantly decreased total [Mg2+] from 17.0 to 16.2 umol/ml. From the citrate/isocitrate ratio, free intracellular [Mg2+] was 0.36, 0.44 and 0.04 umol/ml for 0, 1.2 and 5 mM Pi. The calculated intracellular pH from tissue HCO3- was 6.98, 7.19 and 7.15 respectively. As Pi was increased from 0 to 5 mM, the [Cl-] decreased from 51.7 to 11.6 umol/ml. In contrast, intracellular [K+] was invariant at 128 to 132 umol/ml giving a membrane potential of about -83 mV. The intracellular [Na+] with increasing Pi was 1.62, 3.68 and 2.03 umol/ml. The cytosolic [ATP]/[ADP][Pi] from 3PGK-GAPDH-LDH equilibria decreased from 9,300 M-1 to 3,360 M-1 in the 0 mM and 5 mM Pi perfused hearts. The free energy of ATP hydrolysis was found to be -13.1, -13.3 and -13.2 kcal/mole for 0, 1.2 and 5 mM Pi hearts. The energy of the plasma membrane gradients of [Na+]o3[K+]i2[Cl-]o/[Na+]i3[K+]o2[Cl-]i were +12.7, +11.3 and +13.2 kcal/mole, or 97%, 85% and 99% of the cellular phosphorylation potential. It was concluded that the extent and direction of the major inorganic ions and membrane potential across constitute a Gibbs-Donnan equilibrium system catalyzed by transport enzymes sharing common substrates. The chemical and electrical energies of those gradients are equal in magnitude and opposite in sign to the chemical energy of ATP hydrolysis.

Project Description:

Investigators:

T. Masuda	Visiting Fellow	LMMB, NIAAA
G.P. Dobson	Visiting Associate	LMMB, NIAAA
K. Sato	Visiting Fellow	LMMB, NIAAA
R.L. Veech	Chief	LMMB, NIAAA

Objectives:

To study the factors that regulate the extent and direction of ion gradients across the plasma membrane in the isolated working rat heart. Changes in ion gradients were induced either by (1) changing extracellular Pi concentration or (2) by the addition of 75 mM ethanol into the perfusion medium (study still in progress). Work and oxygen consumption was quantified as was the G of ATP hydrolysis, the cytosolic phosphorylation and redox potentials.

Methods Employed:

Hearts are perfused in the working mode at the NIAAA laboratory. Ion analysis are carried out by atomic absorption. Routine enzymatic analysis is performed at the LMMB, NIAAA.

Major Findings:

The major findings of the study were that the extent and direction of the plasma membrane gradients of Na^+, K^+, Cl^-, Mg^{2+}, HPO_4^{2-} and H^+ in working rat hearts behave as a Gibbs-Donnan near-equilibrium system and are determined primarily by the cytosolic phosphorylation potential.

Significance to Biomedical Research and the Program of the Institute:

To our knowledge, this is the first study using fundamental thermodynamic principles to investigate ion distribution across the plasma membrane. The significance to the Institute relates to the study of the effects of ethanol on the ion distribution and performance in the working rat heart.

Proposed Course:

The proposal that the ion gradients in the heart behave as a simple Gibbs-Donnan near-equilibrium system, in our opinion, will generate much interest in the field of membrane physiology. This is the first time such a system has been proposed for the complex myocardial cell and future work will extend them, particularly the effect of ethanol as there is a vast literature on the effects of ethanol on ion distribution in a variety of organs. It is our aim to quantitate the effect of alcohol of the various ion transporters and extend these findings to investigate the ion distribution across the mitochondrial membrane and its implications to the control of oxygen consumption. Preliminary observations, in a study currently being carried out in our laboratory, shows that while 75 mM ethanol has little affect on the myocardial oxygen consumption, it has a striking effect to decrease the work of the heart by some 40%. The cause of this loss of pump performance is unknown. Analyses are underway to determine the relationship between distribution of ions across the plasma and mitochondrial membranes and energetics in the rat heart perfused with 75 mM ethanol.

Publications:

Masuda T, Dobson, GP, Veech RL. The Gibbs-Donnan Near-Equilibrium System of Heart, J Biol Chem 1991;265:20321-34.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00024-13 LMMB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Genetic and Metabolic Studies of Human Alcoholics

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	R.L. Veech	Chief	LMMB, NIAAA
Others:	J.P. Casazza	Research Chemist	LMMB, NIAAA

COOPERATING UNITS *(if any)*
Department of Academic Medicine, London, England (M. Morgan)

LAB/BRANCH
Laboratory of Metabolism and Molecular Biology

SECTION
Metabolic Control

INSTITUTE AND LOCATION
NIAAA, 12501 Washington Avenue, Rockville, MD 20852

TOTAL MAN-YEARS: 0.7	PROFESSIONAL: 0.7	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In three separate studies involving three different sets of collaborators, elevated levels of 2,3-butanediol has been found in 80% of the blood samples taken from chronic alcoholics when consuming distilled spirits, but not in social drinkers consuming distilled spirits. 2,3-butanediol has also been identified in two other abnormal metabolic states, patients with congenital propionic and methylmalonic acid uria (Duran M, et al. Clin Chim Acta 1987;165:197-204) and premature infants (Mills GA, Walker V. Clin Chim Acta 1989;179:51-9).In rat, three experimental models for the production of 2,3-butanediol have been demonstrated. The first involves elevated blood acetaldehyde entering the brain with an active pyruvate dehydrogenase multi-enzyme complex where it condenses with hydroxyethylthiamine pyrophosphate to form acetoin (Veech RL, et al. Curr Top Cell Regul 1981;13:151-79). The acetoin is subsequently converted in liver to 2,3-butanediol. In the second animal model, 2,3-butanediol in the rats is produced by acetone feeding (Casazza JP, et al. J Biol Chem 1984;259:231-6). Prolonged fasting, which results in elevated serum acetone, does not result in 2,3-butanediol production in man. A third model results in the production of 2,3-butanediol after acute administration of short and medium chain fatty acids. The mechanism of production in this case appears to be similar to that observed in acetaldehyde stimulated production of 2,3-butanediol, but in this case pyruvate serves as both the initial substrate and as the acceptor of the hydroxyethyl moiety due to a decreased level of free CoA. Whether 2,3-butanediol production is due to expression of an aberrant gene product or is due to some other metabolic change caused by chronic ethanol consumption is not known, but the presence of D/L diastereomer of this compound during periods of ethanol ingestion in approximately 40% of all alcoholics prior to the onset of alcoholic liver disease and approximately 25% of all alcoholics with alcoholic cirrhosis in the absence of recent ingestion of ethanol makes this compound a useful indicator of alcoholic liver disease.

Project Description:

Investigators:

R.L. Veech	Chief	LMMB, NIAAA
J.P. Casazza	Research Chemist	LMMB, NIAAA
M. Morgan	Professor	Dept. Academic Medicine, London, England

Objectives:

The purpose of this series of studies was to determine aberrant metabolic pathways which produce 1,2-propanediol, D,L-2,3-butanediol and meso-2,3-butanediol. The hypothesis to be tested thus derives from the classical medical paradigm and seeks to define a certain subset of people with alcohol problems who may be classified as being addicted to alcohol or are alcoholics.

Once abnormal or unusual metabolic consequences were identified within the alcoholic population, it followed that (1) accurate methods for measuring these compounds needed to be developed, (2) whether or not the metabolite identified was produced because of an aberrant enzyme or gene product needed to be established, and (3) the aberrant enzyme or gene product responsible for the metabolic difference needed identification. With accomplishment of these goals it would be possible, given an appropriate setting, to achieve a rational biochemical basis for the diagnosis of alcohol addiction by objective laboratory means and to determine whether these findings are a cause or a result of alcoholism.

Methods Employed:

Identification of the abnormal metabolites was accomplished on contract (Borrison and JTC) using GC mass spectrometry. Measurement of diol was accomplished by use of a Varian gas chromatograph with electron capture detection. Acetol was measured as the dinitrophenylhydrazone derivative by HPLC. Synthesis of chemical intermediates of the diol pathway was accomplished by standard techniques of organic chemistry.

Major Findings:

Clinical Studies: Previous studies have shown blood 2,3-butanediol in 70% of blood samples and blood 1,2-propanediol in 90% of all samples drawn from alcoholics without liver disease when intoxicated (Rutstein DD, Veech RL, Nickerson RJ, Felver ME, Vernon AA, Needham LL, Kishore P, Thacker SB. Lancet 1983;ii:534-6). In alcoholics without liver disease as serum ethanol decreased so did the level of 2,3-butanediol. In most cases 2,3-butanediol was not measurable 32 hours after admission to a detoxification center (Veech RL, Felver ME, Lakshmanan MR, Huang M-T, Wolf S. In: Estabrook R, Srere P eds. Current topic in cellular regulation. New York: Academic Press 1981;17:151-79). No 2,3-butanediol was measurable in control subjects in the absence of ethanol. In a more recent study, ethanol-free blood samples were drawn from 50 normal reference subjects, 35 patients with alcoholic fatty liver, 8 patients with alcoholic hepatitis, 49 patients with alcoholic cirrhosis and 69 patients with non-alcoholic liver disease. 1,2-Propanediol was found in 6% of the reference samples, 11% of the patients with alcoholic fatty livers, 38% of the patients with alcoholic hepatitis, 44% of the patients with alcoholic cirrhosis and 49% of the patients with non-alcoholic cirrhosis. Similarly, meso-2,3-butanediol was found in 11% of the patients with alcoholic fatty livers, 38% of the 8 patients with alcoholic hepatitis, and 44% of the 48 patients with alcoholic cirrhosis. Administration of distilled spirits to 32 reference subjects and 10 subjects with non-alcoholic cirrhosis resulted in a significant increase in the levels of serum 1,2-propanediol. Based on this data, it appears the presence of 1,2-propanediol, previously found in blood drawn from alcoholics who were drinking distilled spirits (Rutstein et al. Lancet 1983;ii:534-6), was the result of ethanol raising 1,2-propanediol levels in a population capable of producing 1,2-propanediol due to pre-existing liver disease. Administration of distilled spirits did not result in an increase in serum meso-2,3-butanediol.

D,L-2,3-butanediol was a more specific marker of alcoholic disease. D,L-2,3-butanediol was present in ethanol-free blood samples from none of the normal reference group, 3% of the patients with alcoholic fatty liver, 23% of the patients with alcoholic cirrhosis and 3% of the patients with non-alcoholic liver disease. The incidence of this compound within each group was independent of commonly used blood tests for liver disease. Administration of distilled spirits to 32 control subjects and 10 patients with non-alcoholic liver disease under controlled conditions did not result in any measurable D,L-2,3-butanediol. There was no difference in the incidence of D,L-2,3-butanediol in patients with alcoholic cirrhosis between those that had been abstinent two weeks or more and those that had not. In fact, the highest level of D,L-2,3-butanediol was identified in a patient who had been ethanol abstinent for 5 years. Clearly, despite the association of D,L-2,3-butanediol with alcoholism, the production of D,L-2,3-butanediol in these patients is not directly related to either ethanol metabolism or its metabolic products.

The source of 1,2-propanediol, meso-2,3-butanediol and D,L-2,3-butanediol and D/L-2,3-butanediol in patients with alcoholic liver disease is not known. The persistent presence of meso-2,3-butanediol and D/L-2,3-butanediol in ethanol abstinent patients with alcoholic liver disease and their absence in a large reference population indicates that the presence of these compounds in these individuals is not due to ingestion of exogenous 2,3-butanediol. Although portal vein shunting in patients with both alcoholic and non-alcoholic cirrhosis could result in an increase in the level of bacterial fermentation products in blood, the data seem inconsistent with gut fermentation being the source of both of these diols. Rather, the presence of D/L-2,3-butanediol in patients with alcoholic cirrhosis, and its absence in patients with non-alcoholic cirrhosis suggest that the production of this compound is due to human metabolism. Serum acetone was found to be elevated in patients with alcoholic liver disease, median 76 (range, <20-770) μ M compared to patients with non-alcoholic cirrhosis, median <20 (<20-191)μ M, and a normal reference population, median 34 (range <20-330) μ M. In addition, serum acetol, a product of acetone metabolism, was significantly correlated ($P<0.01$) with the presence of 1,2-propanediol in ethanol abstinent patients with alcoholic liver disease suggesting that acetone metabolism may contribute to the production of 1,2- propanediol in alcoholics.

Metabolic Studies:

Recent reports have shown the excretion of meso-2,3-butanediol and D/L-2,3-butanediol in urine from children with congenital methylmalonic and propionic acid uria (Duran M, Ketting D, Brinvis L, Dorland L, Wadman SK, Bakkeren JAJM. Clin Chim Acta 1987;165:197-204) and in premature infants (Mills GA, Walker V. Clin Chim Acta 1989;179:51-9) supporting the hypothesis that a pathway distinct from ethanol metabolism can result in the production of these compounds in humans. We have found that meso-2,3-butanediol and D/L-2,3-butanediol are also present in neonatal rats. Schrieber et al. (Schrieber G, Kohlhaw G, Goedde HW, Holzer H. Biochem Z 1963;339:83-90) showed that incubations containing only pyruvate and mammalian pyruvate dehydrogenase produce acetoin. In the absence of the normal acceptor of the acetyl group, coenzyme A (CoA), pyruvate serves as an alternate acceptor with acetoin and CO2 being produced. We have found that injection of short and medium chain fatty acids results in production of up to 0.1 mM serum 2,3-butanediol within one hour of injection with approximately 90% of the butanediol observed being the meso- isomer. The amount of meso-2,3-butanediol produced appears to correlate with a dramatic increase in the level of odd and medium chain acyl CoA intermediates and presumably a decrease in the level of free CoA. These data suggest that the 2,3-butanediol production in these animals may occur by a mechanism similar to that proposed by Holtzer et al. Such a mechanism could also explain the production of 2,3- butanediol by patients with propionic and methylmalonic aciduria (Casazza JP, Song BJ, Veech RL. Trends Biochem Sci 1990;15:26-30).

New methods for the measurement of 1,2-propanediol, D,L-2,3-butanediol, *meso*-2,3-butanediol and acetoin in small volumes of blood have allowed arterio-venous difference studies do be done in both acutely and chronically cannulated animals. In acetone-treated animals and in meal-fed animals injected with short chain fatty acids the largest increase in the level of meso-2,3-butanediol was observed in the portal vein. Prior to treatment with acetone and short chain fatty acids portal vein meso-2,3-butanediol concentrations were at least 10 fold lower than after experimental treatment.

Significance to Biomedical Research and the Program of the Institute:

The specificity of D,L-2,3-butanediol to alcoholic disease makes this compound of particular interest to alcohol research. Even if D/L-2,3-butanediol is not a genetic indicator of alcoholism, a metabolic indicator of excessive alcohol consumption would be invaluable as a diagnostic tool for alcoholism. The close correlation between the incidence of 1,2-propanediol and meso-2,3-butanediol and liver disease suggests that understanding the mechanism of production of this compound may help explain the causes of liver disease.

Proposed Course:

Work in the Laboratory of Metabolism and Molecular Biology has identified D,L-2,3-butanediol as a specific indicator of alcoholic disease in both the absence and the presence of ethanol. In collaboration with the Royal Free Hospital further studies into the production of 1,2-propanediol, D,L-2,3-butanediol and meso-2,3-butanediol are in progress. Studies in sterile animals are currently being done to determined in gut flora are responsible for the production of *meso*-2,3-butanediol in acetone treated and valerate injected animals. Preliminary work indicates that labeled pyruvate contributes to the production of meso-2,3-butanediol in these animals. Further studies into the mechanism of production of this compound in humans using the analytical techniques developed for metabolic analysis and the techniques of molecular biology should give an insight into the etiology of alcoholism and liver disease.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AA 00049-02 LMMB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Enzymes and Metabolites of the Purine Pathway in Rat Liver

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	R.L. Veech	Chief	LMMB, NIAAA
Others:	M.T. King	Research Chemist	LMMB, NIAAA
	J.V. Passonneau	Research Chemist	LMMB, NIAAA

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Metabolism and Molecular Biology

SECTION
Metabolic Control

INSTITUTE AND LOCATION
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TOTAL MAN-YEARS: 1.6	PROFESSIONAL: 1.6	OTHER:

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The metabolism of purine in liver is being investigated, particularly in regard to the effects of ethanol administration. In man, alcohol ingestion or infusion causes elevated uric acid production and hyperuricemia. These changes are accompanied by elevated acetate and phosphate concentrations in the blood, and elevated acetate, phosphate and pyrophosphate contents in rat liver. We are investigating in rats, the effect of ethanol administration, given in the absence or presence of an inhibitor of alcohol dehydrogenase; the effects of the metabolic product acetate; and the effects of phosphate, the plasma concentration, of which is known to fluctuate with ethanol ingestion. These compounds are administered to rats and the livers quick-frozen after 30 minutes. We have completed a study which focussed on the salvage of purines: the enzymes of the metabolic pathway, the carbohydrate compounds, coenzymes, purine bases and nucleosides. The methods for measurement of enzyme activities, and the purine compounds have been newly developed and validated. Further studies are being pursued concerning the changes in free [Mg2+] in lever observed after ethanol treatment of rats as indicated by the citrate/isocitrate ratios.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description:

Investigators:

R.L. Veech	Chief	LMMB, NIAAA
Y.A. Kim	Visiting Fellow	LMMB, NIAAA
M.T. King	Research Chemist	LMMB, NIAAA
J.V. Passonneau	Research Chemist	LMMB, NIAAA
W. Teague	Chemist	LMMB, NIAAA

Objectives:

These studies are for the purpose of systematically evaluating the complex pathway of purine metabolism in liver, and to determine the effects of ethanol on the rate, direction and possible alteration in end products. Previous studies have shown that ethanol consumption results in increased uric acid in blood, uric acid turnover and hyperuricemia. It has been observed that ethanol injection increases P-ribosylpyrophosphate (PRPP) levels in liver, with a presumed increase in purine synthesis.

It is recognized that ethanol raises acetate concentration in the blood; consequently we are investigating whether acetate is responsible for some of the metabolic effects of ethanol. A known inhibitor of ethanol metabolism, 4-methylpyrazole has been administered simultaneously with ethanol to distinguish between the effects of ethanol and its metabolic products.

Ethanol and acetate also elevate pyrophosphate (PPi) concentrations, which is involved in the purine salvage pathway, including the adenosine P-ribosyltransferase (APRT) and guanine (hypoxanthine) P-ribosyl transferase steps. Finally, elevated phosphate levels occur in the serum of alcoholics, followed by hypophosphatemia. The metabolic effects are presumably related, since administration of Pi to rats increases Ppi, PRPP, and ribose-5-P (R5P) in the liver.

Critical to the investigation of these effects is an examination of the enzyme steps involved. The thermodynamic equilibria of several key enzymes have been ascertained, including PRPP synthase and the nucleobase transferases. The measurement of the activities of enzymes involved in the purine pathway under approximate `in vivo' conditions, including the determination of the kinetic dissociation constants is in progress. Such measurements will enable an estimation of maximum flux under measured in vivo conditions.

We hope to define the control of purine metabolism in both de novo and salvage pathways and to focus on aberrations indicated by ethanol administration.

Methods Employed:

Rats are given (ip) ethanol (70 mmoles/kg); 4 methylpyrazole (4MEP, 10 uM); inorganic phosphate (10 and 20 mmoles/kg); or acetate (20 mmoles/kg) and the livers quick frozen with Wollenberger tongs 30 min later. Perchloric acids extracts of the tissue are made by standard procedures. Analyses for metabolites of the glycolytic, citric acid cycle and pentose pathway are made by enzymatic procedures. New radiometric methods have been developed for the measurement of PRPP and R5P. Nucleoside, nucleotides, and base content of the extracts are determined by HPLC.

For the assays of enzyme activities, separate homogenates separate extracts are prepared in a buffer solution designed to preserve activity. The determination of enzyme activities is accomplished by fluorometric and/or radiometric procedures.

Major Findings:

Ethanol administration resulted in the elevation of fructose-6-P, and the increases in ribose-5-P concentration could be almost exactly predicted from the fructose-6-P and glyceraldehyde-3P levels, using the known equilibria of the relevant steps in the pentose phosphate shunt. The formation of the ribose-5-P is thus dependent on the supply of the glucose-P/fructose-6-P, which in turn depends on the glucose supply. The ribose-5-P concentration determines the flux through P-ribosylpyrophosphate synthetase, and the formation of P-ribosyl pyrophosphate, which is essential for both the de nova and salvage pathways of purine metabolism. The administration of 4-methylpyrazole (an alcohol dehydrogenase inhibitor) with ethanol prevented the elevation of ribose-5-P. Further studies will be necessary to explain these results concerning the block between ribose-5-P and P-ribosylpyrophosphate in the presence of the inhibitor. One possibly is the 4-methylpyrazole itself forms a "nucleotide" with P-ribosylpyrophosphate.

In addition to the enzyme measurements reported last year (P-ribosyl pyrophosphate synthetase, hypoxanthine (guanine) phosphoribosyl transferase, adenine P-ribosyl transferase, 5'nucleotidase, purine nucleoside phosphorylases, we have added xanthine dehydrogenase, hypoxanthine dehydrogenase, and adenosine kinase.

The enzyme with the lowest in vitro maximal velocity was the initial step in purine synthesis or salvage, P-ribosyl pyrophosphate synthetase; the activity was one-half that of the next most active enzyme, adenine P-ribosyl pyrophosphate transferase. When the activities of the enzymes in the tissue were calculated using Michaelis-Menten kinetics and the metabolite values measured in the liver, the synthetase activity was the lowest by an order of magnitude. The implications are strong that the synthetase step is rate-limiting and thus controls both the de nova and the purine salvage pathways.

Significance to Biomedical Research and the Program of the Institute:

Purine metabolism is of profound significance in metabolic disease, the most relevant of which is gout which occurs in a portion of the patients who have elevated blood urate levels. Increased urate concentrations in blood can occur as a result of ethanol ingestion, and thus lead to a clinical manifestation of gout. Other diseases associated with abnormal purine metabolism not necessarily directly connected with ethanol metabolism are: the Lesch-Nyhan syndrome, which hypoxanthine (guanine) phosphoribosyl transferase deficiency, which results in the formation of kidney stones; adenosine deaminase/purine nucleoside phosphorylase deficiencies which result in immunodeficiencies; and hereditary xanthinuria, which causes xanthine calculi in the urinary tract, and defects in molybdenum metabolism, which in turn causes neurological symptoms. All of these diseases could be exacerbated by excessive ingestion of ethanol, which would increase flux through the purine pathway, and the accumulation of products where enzyme deficiencies exist.

Proposed Course:

The methodology to pursue studies on purine metabolism is now well in hand. The effects of ethanol, which would stress a system with a low rate of flux (compared with the glycolytic pathways, both oxidative and anaerobic) will aid in unraveling the control and possible treatment of defects in purine metabolism.

Publications:

Kim Y-A, King MT, Teague WE, Rufo GA, Veech RL, Passonneau JV. The Regulation of the Purine Salvage Pathway in Rat Liver, A J Physiol, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00057-01 LMMB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Effects of Ethanol on Natural Killer (NK) Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	R.L. Veech	Chief	LMMB, NIAAA
Others:	L. Albornoz	Visiting Fellow	LMMB, NIAAA

COOPERATING UNITS *(if any)*
Johns Hopkins University, Baltimore, MD (E. Mezey)

LAB/BRANCH
Laboratory of Metabolism and Molecular Biology

SECTION
Metabolic Control

INSTITUTE AND LOCATION
NIAAA, 12501 Washington Avenue, Rockville, MD 20852

TOTAL MAN-YEARS: 0.1	PROFESSIONAL: 0.1	OTHER:

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose of this study is to determine whether the exposure to acute and/or chronic ethanol consumption increases natural killer (NK) activity of immune effector cells. NK activity is a possible mechanism of ethanol induced liver damage. Acute exposure to ethanol has been reported by others as inhibitory of NK activity in vitro. However, we have found that chronic ethanol increases NK activity in vivo in rat pit cells, which are NK cells resident in the liver. Ethanol might be implicated in the stimulation of NK activity of immune effectors against liver cells. In this study, we have isolated pit cells (Large granular lymphocytes) from Lewis rat hepatic sinusoids, and determined an increase in their ability to bind to, and lyse specific target cells, including an endothelial cell line (SVEC4- 10), when the rats have been treated with ethanol for two weeks. Both vascular and sinusoidal endothelial cells, as well as liver parenchymal cells, are potential target cells for these NK effectors. In order to assess this finding in human immune effector cells, we will examine the NK activity of human circulating lymphocytes, in each of the following groups of patients: a- Chronic alcoholics who are still drinking; b- Chronic alcoholics recently withdrawn from dependency; c- sober alcoholics (alcoholics with no recent drinking history); and d- Normal healthy volunteers. We will measure the effector- to- target binding ratio, the effector- to- target lysing ratio, by chromium release assays. Several target cells will be utilized, including human endothelial cell lines as well as hepatoma cell lines. We will measure perforin (pore forming protein/ cytolysin) mRNA levels in the lymphocytes by molecular probing. We intend to verify the involvement of perforin as the mediator of increased NK activity, and correlate the increase in binding capacity and lysing activity with perforin mRNA levels in the effector cells. The effect of ethanol on circulating human lymphocytes has been determined, but not in correlation with perforin levels. The implication of this cytolytic protein as the specific mechanism of liver cell killing has clinical relevance in regards to the potential inhibition of liver cell damage by ethanol, as to what pertains to NK cell mediated damage.

Project description:

Investigators:

R.L.Veech	Chief	LMMB, NIAAA
L. Albornoz	Visiting Fellow	LMMB, NIAAA
E. Mezey	Professor	JHU

Objectives:

From rat liver sinusoidal lymphocytes (pit cells), and human circulating NK lymphocytes, measure and correlate:

1- effector to target binding capacity (chromium release assay)
2- effector to target lytic activity (chromium release assay)
3- perforin mRNA levels in the effector cell population

Methods employed:

Enzymatic perfusion and centrifugal elutriation for liver cell isolation, ficoll gradient separation for liver cell and circulating lymphocyte isolation. Four hour chromium release assays, molecular probing for mRNA levels.

Significance to Biomedical Research and the Program of the institute:

Alcoholic hepatitis is a known consequence of ethanol consumption. Given our previous finding that chronic ethanol increases NK activity, natural killer cells may play a major role in the liver damage in alcoholics. If perforin is proven to be the main mechanism of cell mediated liver cell damage in alcoholic hepatitis, this may have important implications in its therapeutic approach.

Proposed course:

Measure binding capacity, lytic activity and perforin mRNA content of circulating lymphocytes in drinking alcoholics, acutely withdrawing alcoholics, sober alcoholics, and normal healthy controls.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00036-05 LMMB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Regulation of ethanol-inducible cytochrome P450 gene (IV)

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	B.J. Song	Section Chief	LMMB, NIAAA
Others:	J.P. Casazza	Research Chemist	LMMB, NIAAA
	M.V. McGuire	Biologist	LMMB, NIAAA
	K.S. Park	Visiting Fellow	LMMB, NIAAA
	D.H. Sohn	Visiting Fellow	LMMB, NIAAA
	R.L. Veech	Chief	LMMB, NIAAA

COOPERATING UNITS *(if any)*
Department of Pediatrics, Albert Einstein Medical School, New York, New York. (Dr. P. Saenger)

LAB/BRANCH
Laboratory of Metabolism and Molecular Biology

SECTION
Molecular Genetics

INSTITUTE AND LOCATION
NIAAA, 12501 Washington Avenue, Rockville, Maryland 20852

TOTAL MAN-YEARS: 3.0	PROFESSIONAL: 3.0	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

As a continuous ongoing project, the molecular regulatory mechanism of P450IIE was further investigated. We have previously identified and determined the structures of the ethanol-inducible cytochrome P450 (P450IIE) of both rat and human. We have also demonstrated three distinct types of regulation of P450IIE. The mechanism of P450IIE mRNA stabilization was further studied to identify intermediary metabolites responsible for the mRNA stabilization. The level of two ketone bodies (β-hydroxybutyrate and acetoacetate) positively correlated with the elevated level of P450IIE mRNA. In addition, the molecular mechanism of P450IIE reduction by CCl_4, was also examined. Aniline hydroxylase and the amounts of immunoreactive P450IIE were rapidly decreased in a time-dependent manner after CCl_4 injection. These declines were not accompanied by the changes in the level of P450IIE mRNA and transcriptional activity, indicating a post-translational inhibition by CCl_4 probably due to specific destruction of P450IIE by locally generated free radical metabolites. In contrast, the amounts of immunoreactive other P450s such as P450IA and P450IIC did not appear to be affected by CCl_4, indicating a specific destruction of P450IIE by its substrate. A method for the measurement of P450IIE in easily obtainable human tissues was also established. P450IIE1 expressed in cultured lymphocytes could be easily detected by specific antibody to P450IIE. The induction of lymphocyte P450IIE was observed in both human and rat lymphocytes. The levels of P450IIE in lymphocytes from alcoholic patients at admission and discharge are being studied.

Project Description:

Investigators:

B.J. Song	Section Chief	LMMB, NIAAA
J.P. Casazza	Research Chemist	LMMB, NIAAA
M.V. McGuire	Biologist	LMMB, NIAAA
K.S. Park	Visiting Fellow	LMMB, NIAAA
P. Saenger	Physician	Einstein Med School
D.H. Sohn	Visiting Fellow	LMMB, NIAAA
R.L. Veech	Chief	LMMB, NIAAA

Objectives:

1) We have reported P450IIE induction via mRNA stabilization in starved and diabetic animals. The mechanism of mRNA stabilization was further studied to identify intermediary metabolites responsible for P450IIE mRNA stabilization. 2) Despite numerous reports on the multiple mechanisms of P450IIE induction, the mechanism of its reduction by substrate, for instance, CCl_4, has not been investigated on a molecular level. The molecular mechanism of suicidal destruction of P450IIE by CCl_4, a substrate of P450IIE, was further examined whether P450IIE suppression by CCl_4 was related to transcriptional inhibition.

3) P450IIE, inducible by ethanol and other compounds, is believed to be involved in the metabolism of various substrates including potent carcinogens and several hepatotoxic agents. Since its levels, fluctuating with the status of pathophysiological conditions, predispose individuals to certain adverse reactions, it is often desirable to determine its level accurately. A specific method was developed to analyze P450IIE in easily obtainable tissues was developed.

Methods Employed:

Rats were treated with a high fat diet in order to simulate the conditions of starved or diabetic animals. As positive controls, different groups of rats were also treated with ethanol and acetone, respectively. The levels of P450IIE-mediated catalytic activity and the amounts of immunoreactive P450IIE protein and its mRNA were measured. The levels of three ketone bodies and hepatic P450IIE mRNA were measured for the individual animal to obtain the relationship between the two parameters. In order to elucidate the mechanism of P450IIE reduction by its own substrate, CCl_4, the microsomal fractions of rats treated with CCl_4 for different times were prepared by the method of differential centrifugation. The washed microsomes were used for P450IIE-associated enzyme activities and immunoblot analysis. The total cellular RNA from the same groups were also prepared for the quantitation of P450IIE mRNA by Northern blot analysis. The lymphocytes from human diabetic patients and alcoholic patients were prepared from circulating blood and grown in culture system in the presence of mitogens. The lymphocytes were maintained for 4 to 5 days with a change of culture media at day 3. The levels of P450IIE1 in the whole homogenate of lymphocytes (1 to 2 mg) were determined by Western immunoblot analysis following SDS-polyacrylamide gel electrophoresis. The band intensity of immunoreactive P450IIE was determined by a densitometer and compared to the values of other blood chemistry.

Major Findings:

(1) P450IIE mRNA Stabilization by a High Fat Diet.

The levels of aniline hydroxylase and immunoreactive P450IIE were elevated in both acetone- and high fat-treated animals. However the P450IIE mRNA was only elevated in animals treated with high fat diet but not with acetone or ethanol treatment. The elevation of P450IIE mRNA noted in individual animal positively correlated ($r = 0.76$) with the individual levels of serum acetoacetate and β-hydroxybutyrate which were elevated in the high fat-treated animals. In contrast, the increases in P450IIE mRNA did not correlate with the level of serum acetone elevated in acetone-treated rats although the amounts of immunoreactive P450IIE are increased. The negative result of nuclear run off transcription assay further indicated that P450IIE in high fat diet-treated animals may be induced by a similar mechanism of induction (mRNA stabilization) already observed in starved or diabetic animals.

(2) Post-Translational Inhibition of P450IIE by CCl_4.

The molecular mechanism of P450IIE inhibition by its own substrate, CCl_4 was studied by measuring its enzyme activity and the amounts of immunoreactive protein and mRNA. The P450IIE-associated enzyme activity, aniline hydroxylase, was rapidly decreased within 2 h after CCl_4 treatment. The decrease in aniline hydroxylase was time-dependent and mainly observed in hepatic tissue. No differences were noticed in the amounts of immunoreactive P450IIC and P450IA despite significant decreases in their respective catalytic activities, indicating a specific decrease of P450IIE protein by its substrate. However, the decreases in P450IIE enzyme activity and immunoreactive protein by CCl_4 were not accompanied by a decline in its mRNA level nor changes in nuclear transcription activity. The data thus suggested a post-translational reduction of P450IIE by CCl_4, probably due to specific reduction of the P450IIE protein by its own substrate.

(3) Induction of P450IIE in Fresh and Cultured Lymphocytes.

Based on the data of P450IIE regulation in animal models, we have studied to determine whether P450IIE can be induced in human tissues that are easily obtainable. The levels of P450IIE in fresh and cultured lymphocytes were determined by immunoblot analysis using polyclonal antibodies to P450IIE. The levels of P450IIE in lymphocytes from some of the diabetic patients were elevated than those of the healthy volunteers. The degree of P450IIE induction in diabetic patients correlated with the levels of hemoglobin A_{1c}, a metabolic indicator for these patients ($r = 0.87$). The presence and induction of P450IIE were also detected in fresh lymphocytes isolated from starved animals. Although four to five fold elevation in the levels of P450IIE in cultured lymphocytes from alcoholic patients than those of control subjects were initially observed, the levels of P450IIE in fresh lymphocytes are being determined by the same method.

Significance to Biomedical Research and the Program of the Institute:

We previously cloned cDNA clones for both human and rat P450IIE and demonstrated three distinct types of induction of P450IIE: transcriptional activation, mRNA stabilization, and protein stabilization. During the last year, we identified putative metabolites which may be responsible for the elevation of P450IIE mRNA stabilization. We also revealed another mechanism of P450IIE regulation via post-translational destruction of P450IIE by CCl_4. The metabolism of CCl_4 was reported to be catalyzed by the isozymes of cytochromes P450 which are induced by ethanol and phenobarbital treatment. It was precisely determined that P450IIE is the major enzyme involved in the biotransformation of CCl_4. The specific down-regulation of P450IIE by CCl_4 provides additional mechanism of regulation in P450IIE along with four or five types of regulatory mechanisms on P450IIE. The multiple types of regulation on P450IIE provides an unique example for not only in the area of P450s most of which are induced by transcriptional activation, but also sets an interesting example of a protein regulation in general. It was suggested from our laboratory that ethanol-inducible P450 (P450IIE) may be responsible for the production of 1,2-propanediol whose levels are elevated in the sera of alcoholics. In order to study the relationship between the levels of 1,2-propanediol and P450IIE, we need to develop a method to determine the relative level of P450IIE expressed in human tissues. The detection of P450IIE in human lymphocytes and its induction in some patients proves to be the first demonstration. Furthermore the immunological method provides an important way of examining clinical specimens which may be extremely useful for clinical aspects of diagnosis and prognosis of certain disease states such as diabetes and alcoholism.

Proposed Course:

Our data on P450IIE in lymphocytes are being compared to other known methods useful to diagnose of alcoholism or alcohol abuse. The P450IIE protein purified from cultured human lymphocytes will be subjected to amino acid sequence determination to get an information about its structure potentially needed for the estimation its catalytic activity as compared to hepatic P450IIE. The levels of P450IIE in lymphocytes from human alcoholic individuals will be examined more extensively and compared to other known methods of identifying state markers of human alcoholism. More sensitive detection method for P450IIE1 in human specimens will be developed.

Publications:

Song BJ, Veech RL, Saenger P. Cytochrome P450IIE is elevated in lymphocytes from poorly controlled diabetes mellitus, J Clin Endocr Met 1990;76:1036-40.

Sohn DH, Yun YP, Park KS, Song BJ. Post-translational destruction of P450IIE by CCl_4, its substrate, Biochem Biophys Res Comm 1991;179:449-54.

Yun YP, Casazza JP, Sohn DH, Veech RL, and Song BJ. Pretranslational activation of cytochrome P450IIE during ketosis induced by a high fat diet, Mol Pharmacol 1991.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00037-05 LMMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Structure and regulation of genes for pyruvate dehydrogenase multienzyme complex (IV)

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.	B.J. Song	Section Chief	LMMB, NIAAA
Others:	J.P. Casazza	Research Chemist	LMMB, NIAAA
	J.W. Huh	Visiting Associate	LMMB, NIAAA
	J.H. Ryu	Visiting Fellow	LMMB, NIAAA
	H.C. Sung	Visiting Associate	LMMB, NIAAA
	R.L. Veech	Chief	LMMB, NIAAA

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Metabolism and Molecular Biology

SECTION

Molecular Genetics

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Rockville, Maryland 20852

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.5	3.5	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

As a continuing project, all the subunits of the pyruvate dehydrogenase (PDH) multienzyme complex were purified to near homogeneity from bovine kidney and heart. The purified proteins were subsequently used for further purification of PDH-kinase and PDH-phosphatase and generation of antibodies against each subunit. Using these antibodies and oligodeoxynucleotides, we have also cloned and determined the nucleotide structures of full-length cDNAs for PDH E1α, E1β, and E3 subunits. We also demonstrated differential expression of these PDH genes in cell- and tissue-specific manners. Particularly an immunologically distinct isoform of PDH E1α with different molecular mass was identified in rat testis. This PDH E1α isoform is only detected by anti-peptide antibodies and immunoprecipitated by monospecific antibodies against PDH E1β subunit, common to PDH complex in all tissues examined but not by those against PDH E1α subunit of kidney. This specific PDH E1α isotype appeared to be present in testis and brain which may support the potential role of PDH isozyme in acetoin, thus butanediol production found in alcoholic individuals as well as in animal models. The coordinate, transcriptional activation of the subunits of the PDH complex during adipocyte differentiation was also demonstrated. PDH-specific protein kinase and phosphatase were also purified to homogeneity and their N-terminal amino acid sequences were determined. Another protein with PDH-kinase activity (M_r 68,000) was also purified: the peptide sequence analysis revealed that this protein is identical with calelectrin. Based on the partial amino acid sequences, several oligodeoxynucleotides were synthesized and used to clone the genes coding for PDH-specific kinase as well as PDH-phosphatase, whose structures and regulation were not characterized yet.

Project Description:

Investigators:

B.J. Song	Section Chief	LMMB, NIAAA
J.P. Casazza	Research Chemist	LMMB, NIAAA
J.W. Huh	Visiting Associate	LMMB, NIAAA
J.H. Ryu	Visiting Fellow	LMMB, NIAAA
H.C. Sung	Visiting Associate	LMMB, NIAAA
R.L. Veech	Chief	LMMB, NIAAA

Objectives:

The specific aims of this project are to investigate the mechanism of acetoin production, precursor of 2,3-butanediol by the PDH complex in a tissue-specific manner as evidenced in animal models. By studying the characteristics of the genes coding for PDH complex at molecular level, we wish to develop cDNA clones and antibodies that can be utilized as potential probes for the genetic studies of human population including alcoholics with high and low levels of 2,3-butanediol. The structural information and differential regulation of the PDH-specific kinase and PDH-phosphatase genes will be studied in the established cells in culture originally obtained from patients with different clinical symptoms.

Methods Employed:

The pyruvate dehydrogenase (PDH) complex would be purified from bovine kidney or heart mitochondria fractions. The two regulatory protein (PDH-kinase and PDH-phosphatase) would be further purified from the purified PDH complex using column chromatography to near homogeneity and subjected to amino acid sequence analyses. Based on data of amino acid sequencing on the purified proteins, oligopeptides will be synthesized to generate anti-peptide antibodies against purified proteins. The anti-peptide antibodies and synthetic oligodeoxynucleotides will be used to screen cDNA libraries of various tissues of several species including human. The cDNA clones for each protein will be used to screen the human genomic DNA library to study the gene structures. Peripheral lymphocytes or skin fibroblasts obtained from human individuals, including alcoholics, and grown in tissue culture will be analyzed for human genetic polymorphism using cloned PDH cDNA probes with appropriate biochemical and immunochemical approaches.

Major findings:

(1) Identification of immunologically distinct PDH E1α isoform in rat testis.

The tissue-specific production of 2,3-butanediol reported from this laboratory indicated a possibility of PDH isoenzyme present in testis and brain. The tissue specific expression of the PDH complex was reexamined by the enzyme activity, immunoreactive proteins, and mRNA expression for its respective subunits. The levels of immunoreactive proteins for PDH E1β, E2, E3 subunits generally corresponded to those of enzyme activities measured for four tissues of brain, heart, kidney, and liver. However, in testis, there was no immunoreactive PDH E1α protein detected by the monospecific polyclonal antibodies against PDH E1α from kidney despite considerable amounts of enzyme activity, suggesting an immunologically distinct PDH E1α isoform present in this tissue. The PDH E1α isoform was further demonstrated by the anti-peptide antibodies against the peptide around two major phosphorylation sites of PDH E1α subunit and immunoprecipitation of the ^{32}P-labeled PDH E1α isoform by the antibodies against PDH E1β subunit which are common in all tissues examined. The PDH E1α isoform appeared to be a larger (M_r 56,000 daltons on SDS-polyacrylamide gel electrophoresis) than the regular counterpart (M_r 41,000 daltons). because of differences in their sizes, we proposed different regulation by allosteric regulators as well as by PDH-kinase. Our recent data also suggest that the PDH complex in rat testis behaves differently from those in other tissues in their purification protocols and different environment.

(2) Purification and characterization of PDH-specific kinase and PDH-specific phosphatase.

Catalytically active PDH-specific kinase with heterodimeric subunits was purified to near homogeneity from bovine kidney. The N-terminal amino acid sequences were determined for both catalytic (PDH-K_c, M_r of monomer 48,000 daltons) and regulatory (PDH-K_r, M_r of monomer 45,000 daltons on a SDS-polyacrylamide gel) subunits of PDH-specific kinase. Internal protein sequences were also obtained for the trypsin- or Arg C-digested peptide fragments of this protein kinase. According to the amino acid sequences, oligopeptides for the respective subunit were synthesized and used for polyclonal antibodies. In contrast, the PDH-specific phosphatase was also purified from bovine heart by using dihydrolipoyl transacetylase as a biospecific ligand for affinity chromatography. The N-terminal amino acid sequence of the PDH-phosphatase (PDH-P_c, M_r of 50,000 daltons) was determined. Since the N-terminal amino acid is blocked for the regulatory subunit of PDH-phosphatase (PDH-P_r, M_r of 90,000 daltons), an internal peptide sequence was being determined. When the frozen heart was used as starting material, another protein (M_r 68,000 daltons) with PDH-phosphatase activity was purified from the same affinity column in Ca^{2+}-dependent manner. However, the amino acid sequences of this monomeric PDH-phosphatase revealed that it is identical with calelectrin, or p68, a major Ca^{2+}-binding protein of a lipocortin family, whose catalytic function has not been known. The specific activity of the monomeric PDH-phosphatase of is about one third of the heterodimeric mitochondrial enzyme (unpublished data).

(3) Cloning of cDNAs for the subunits of the PDH multienzyme complex.

The full-length cDNA clones for PDH E1α, E1β, and E3 subunits were isolated and their nucleotide sequences and chromosomal localization were determined. The DNA and deduced protein structures of PDH E1α in human brain and liver are identical but clearly different from those clones isolated from human foreskin and fetal liver reported recently. Our data thus indicate that the differences in 2,3-butanediol production in two tissues may not due to the structural differences as we originally expected, but may be related to differential regulation altered by metabolic regulators and PDH-kinase or PDH-phosphatase. The full-length cDNA clones for PDH E1α, E1β, and E3 subunits were inserted to a vaccinia virus pSC11 vector for the expression of large quantities of PDH subunits followed by biochemical and kinetic analyses. The differential expression of these PDH subunits were studied in the established fibroblasts originally isolated from patients of varying degree of lactic acidosis. The coordinate, transcriptional activation of the PDH complex during 3T3 adipocyte differentiation was also observed (unpublished data). In addition to the ongoing studies described above, we have initiated other projects of cloning the regulatory enzymes of the PDH complex. Based on the partial amino acid sequences of the enzymes described above, several oligodeoxynucleotides were synthesized and used for the cloning of the cDNAs for PDH-kinase and PDH-phosphatase, respectively. We successfully identify the cDNA clones from bovine and human heart PDH-Kr subunits whose deduced protein sequence matched with the N-terminal amino acid sequence of this subunit (unpublished data). Using two different sets of oligodeoxynucleotides for the PDH-Kc and PDH-Pc subunits, respectively, as primers for the polymerase chain reaction, specific DNAs (ranging 600 and 700 base pairs long) were amplified. The amplified DNA are being subjected to nucleotide sequencing to verify their identities. In addition, the anti-peptide antibodies are being used for the cloning of cDNAs for PDH-Kinase and heterodimeric PDH-Phosphatase subunits.

Significance to Biomedical Research and the Program of the Institute:

The data from our laboratory indicated that 2,3-butanediol, one of the unusual metabolites found in human alcoholics, could be generated by the side reaction of pyruvate dehydrogenase (PDH) located in brain and testis. Recent data also indicate that 2,3-butanediol can still be detected in abstinence people, indicating that the production of this compound may not totally be dependent on the level of circulating acetaldehyde, but rather depend on the individual's genotype. Since it is specifically associated with alcoholic individuals, the latter possibility will be carefully examined to correlate individual genetic differences with the level of 2,3-butanediol to determine its potential as a genetic marker for human alcoholism. It is quite essential to the program of the Institute to study the research problems on human alcoholism by combination of molecular biology and immunochemical techniques. In addition, the characterization of structure and regulation of PDH-kinase and PDH-phosphatase will provide valuable information in not only in the research areas of carbohydrate metabolism and energy production but also in the general protein biochemistry since these key PDH-regulating enzymes represent new classes of serine protein kinase and phosphatase independent from cAMP, cGMP, and calmodulin stimulation. The abnormal regulation of the PDH complex is well known in many pathological conditions including metabolic, neurological diseases, cancer or diabetes. The thorough knowledge on the regulation and gene expression of the PDH subunits will be of benefit to the understanding and lead to potential cures or alleviation of these undesirable conditions.

Proposed Course:

1. The immunologically distinct PDH E1α present in testis will be purified by different chromatography monitoring PDH E1 activity using [1-^{14}C]pyruvate. The biochemical and kinetic properties of the purified enzyme will be compared with those found in other tissues and species. The cDNA for testis PDH E1α

isoform will be isolated and its DNA and deduced protein structures will be compared to those of the liver PDH E1α clone. Both biochemical and molecular biological approaches will demonstrate an isoform of PDH E1α which may be regulated differently (may not be suppressed by metabolic regulators) and be responsible for the 2,3-butanediol production.

2. The full-length cDNA clones for the PDH subunits will be expressed in eukaryotic cell culture system in order to study the characteristics of human enzymes which are very difficult to obtain and to study the exact mechanism of the defect mentioned earlier. It is also aimed to see the production of butanediol from acetoin which is a side product of PDH dependent reaction.

3. We now have several positive cDNA clones for PDH-kinase catalytic subunit. The DNA sequences of these clones will be determined and their deduced protein sequences will be compared to amino acid sequences for this subunit. The oligodeoxynucleotides will be used for identifying cDNA clones for heterodimeric PDH-phosphatase subunits. The anti-peptide polyclonal antibodies will be also used for the screening purposes.

4. We will study genomic organization of the PDH genes that are common to every subunit which may be responsible for their coordinate expression during normal development as well as differentiation. The possibility of genetic polymorphism and differences in abnormal tissues will also be pursued by analyzing restriction fragment length polymorphism for genomic DNAs or specific ribonuclease digestion method in fibroblasts or lymphocytes obtained from human subjects, including alcoholic and lactic acidosis patients.

Publications:

Huh TL, Casazza JP, Huh JW, Chi YT, and Song BJ. Characterization of two different cDNA clones for pyruvate dehydrogenase E1β subunit and differential regulation in cultured fibroblasts, J Biol Chem 1990;265:13320-6.

Huh TL, Chi YT, Casazza JP, Veech RL, and Song BJ. Identical sequences for human brain and liver pyruvate dehydrogenase E1α subunits, Mol Cells 1990;1:74-8.

Huh TL, Huh JW, Ryu JH, Casazza JP, Song BJ, and Veech RL. Characterization of immunologically distinct pyruvate dehydrogenase E1α subunit present in rat testis, J Biol Chem, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00050-02 LMMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The Relationship of Cytokines to Alcoholic Liver Disease

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	R.L. Veech	Chief	LMMB, NIAAA
Other:	J. Jones	Visiting Scientist	LMMB, NIAAA
	L. Albornoz	Visiting Fellow	LMMB, NIAAA
	M. McGuire	Chemist	LMMB, NIAAA
	B.J. Song	Ph.D.	LMMB, NIAAA
	Y.P. Yun	Visiting Fellow	LMMB, NIAAA

COOPERATING UNITS *(if any)*

John Hopkins University, Baltimore, MD (E. Mezey, G. Briefel, J. Anderson)

LAB/BRANCH

Laboratory of Metabolism and Molecular Biology

SECTION

Molecular Genetics

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Rockville, Maryland 20852

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
.1	.1	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose of this study was to determine the role which chronic exposure to ethanol and its metabolic product acetate play in altering the function of the immune system in alcoholics and subjects with alcoholic hepatitis and alcoholic cirrhosis. Earlier we had shown that elevation of plasma tumor necrosis factor α (TNF α) predicted death in patients with severe alcoholic hepatitis. Elevation of no other acute Phase cytokine was found to do so, nor was there a correlation with standard liver function tests, nor with abstinence after discharge from 30 days in hospital. In order to further examine alterations in immune function in alcoholics we determined this year, that plasma levels of interferon γ(IFNγ) were elevated in alcoholic subjects without clinically obvious liver disease during withdrawal from alcohol. Correlated with this elevation of IFNγ, there was the expected increase in the expression of MHC class I antigens circulating mononuclear cells, but a paradoxical decrease in expression of MHC class II protein on the cell surface of circulating leukocytes. Finally, it was determined that end stage renal disease patients, exposed to 1.5 mM acetate but without exposure to ethanol as a precursor showed acute elevation within 4 hours of circulating transforming growth factor β (TGFβ) during hemodialysis. TGFβ is associated with the production of fibrosis and collagen formation, a prominent feature of alcoholic cirrhosis, while TNFalpha is associated with cell death, also a prominent feature of alcoholic cirrhosis and alcoholic hepatitis. This finding of widespread abnormalities in alcoholics begin to present a coherent pattern of alteration in immune function by ethanol and its metabolite acetate which may have clinical significance not only for our understanding of pathogenesis, but also for improved treatment of the severe and often fatal medical consequences of alcoholism. These findings also have implications for HIV-1 infection and progression of disease in a group of poly drug abusers, who also abuse alcohol, and constitute a high risk group for HIV-1 infection.

Project Description:

Investigators:

R.L. Veech	Chief	LMMB, NIAAA
L. Albornoz	Visiting Fellow	LMMB, NIAAA
J. Anderson	Physician	JHU
G. Briefel	Physician	JHU
J. Jones	Visiting Scientist	LMMB, NIAAA
M. McGuire	Chemist	LMMB, NIAAA
E. Mezey	Professor	JHU
B.J. Song	Ph.D.	LMMB, NIAAA
Y.P. Yun	Visiting Fellow	LMMB, NIAAA

Objectives:

The plasma levels of various cytokines such as TNFα, IL-1α and IL-1β and their relationships to alcoholic liver disease, in particular, alcoholic hepatitis were studied.

Methods Employed:

Blood samples from alcoholic hepatitis patients, alcoholic patients without clinically apparent liver disease, alcoholic cirrhosis patients and nonalcoholic healthy controls were collected by venipuncture and plasma was prepared from each sample. In order to measure the plasma levels of TNFα, IL-1α and IL-1β commercially available kit ELISA's (enzyme-linked immunosorbent assay) were used.

Major Findings:

(1) Elevated plasma TNFα levels either at admission or discharge from the hospital are associated with death in 82% (14/17) of alcoholic hepatitis patients.

(2) Absence of elevated plasma TNFα is associated with survival 100% (6/6).

(3) Plasma IL-1α levels are significantly increased in alcoholic hepatitis whereas plasma IL-1 is not.

(4) Neither IL-1α nor IL-1β is correlated with outcome in the alcoholic hepatitis group.

(5) The elevated plasma TNFα levels in those alcoholic hepatitis patients that died suggests an observed first order rate constant for mortality of 0.87/year.

Significance to Biomedical Research and the Program of the Institute:

Severe alcoholic hepatitis is associated with significant mortality. Our observations of decreased long-term survival with the presence of plasma TNFα in alcoholic hepatitis suggests that this cytokine may be an important tool not only for prognosis, but also an important determinant in understanding the pathophysiology of this disease and other forms of highly fatal liver disease. Perhaps with a better understanding of the pathophysiology of alcoholic hepatitis improved therapy can be developed.

Proposed Course:

Investigation into the possible effectors of TNFα production from monocytes and monocytic cells such as Kupffer cells in the hepatic sinusoids will be done. With the elucidation of some effectors of TNFα production we will then study how these effectors cause production of TNFα from these types of cells.

Publications:

Jones JM, Veech RL, Abbassi F, Yu K, Yeralan O, Briefel GR, Anderson J, Mezey E. Altered expression of HLA antigens and CD16 receptors on Leukocytes of alcoholic subjects and uremic patients, Alcoholism: Clin Exptl Study, in press.

Anderson J, Briefel G, Jones JM, Ryu JH, McGuire M, Yun YP. Effects of acetate dialysate on transforming growth factor β_1, interleukin and β_2 microglobulin plasma levels, Kidney Intl, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00038-04 LMMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

NMR Studies of Cerebral Blood Flow and Energy Metabolism

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	A.C. McLaughlin	Section Chief	LMMB, NIAAA
Others:	L. Ligeti	Visiting Scientist	LMMB, NIAAA
	J. Pekar	Senior Staff Fellow	LMMB, NIAAA

COOPERATING UNITS *(if any)*

NMR Center, NIH (C. Moonen); Dir, NINDS (J. Alger); Georgetown University (P. Van Zijl); Children's Hospital of Philadelphia (L. Sutton)

LAB/BRANCH

Laboratory of Metabolism and Molecular Biology

SECTION

Physical Chemistry

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Rockville, Maryland 20852

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.40	0.40	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This study was undertaken to assess a new method for the non-invasive determination of cerebral blood flow without the use of radioactive tracers. Specifically, we used a new 19F NMR technique to measure the clearance of a fluorinated inert gas, CHF3, from the brain. We tested the new technique by comparing the cerebral blood flow values determined by 19F NMR with cerebral blood flow values determined simultaneously by radioactive microsphere techniques. The cerebral blood flow values determined by 19F NMR and radioactive microsphere techniques agreed reasonably well, and showed the same response to variations in the arterial CO2 level. We conclude that the 19F NMR technique gives a quantitative measure of cerebral blood flow.

We used NMR techniques to localize the 19F NMR spectra (and thus the blood flow) to a well defined 4 cc volume in the brain. Using this localization technique, the 19F NMR approach was now non-evasive, and thus applicable to clinical studies. However, there were two drawbacks to this technique. First, the spatial resolution was rather poor, and second, only one region of the brain was investigated at a time.

Both these problems were circumvented by a new technique that was developed in the last year. Instead of obtaining spectra from a single localized region, we used NMR techniques to image the cerebral blood flow over the entire brain. The spatial resolution for the imaging studies (0.8 cc) is much better than the spatial resolution obtained with the previous technique (4 cc).

Project Description:

Investigators:

A.C. McLaughlin	Section Chief	LMMB, NIAAA
J. Alger	Special Expert	Dir, NINDS
L. Ligeti	Visiting Scientist	LMMB, NIAAA
C. Moonen	Director	NMR Center, NIH
J. Pekar	Senior Staff Fellow	LMMB, NIAAA
T. Sinnwell	Biochemist	LMMB, NIAAA
L. Sutton	Professor	Neurosurgery, Children's Hospital of Philadelphia
P. Van Zijl	Assistant Professor	Georgetown University

Objectives:

The first objective of this project is to develop a non-evasive 19F NMR technique for measuring regional cerebral blood flow in animals.

The second objective is to combine the 19F NMR technique for measuring cerebral blood flow with other NMR techniques for determining cerebral bioenergetics (31P NMR) and transmembrane sodium gradients (23Na NMR).

Methods Employed:

Our technique is based on the modified Kety-Schmidt approach, i.e., that blood flow can be calculated from the wash-out curve of an inert diffusible tracer from the brain if the arterial wash-out curve and the brain/blood partition coefficient are also known. We use 19F NMR to measure the washout of the fluorinated inert gas, CHF3, from the brain and the arteries, and to determine the brain/blood partition coefficient.

Physiological parameters, e.g., blood gases, arterial blood pressure, etc., were measured by standard techniques. Surgical procedures were performed using standard approaches.

Major Findings:

Our major finding is that 19F NMR techniques can be used to obtain three-dimensional image of blood flow in the brain, with a voxel size of 0.8 cc. This finding is important for future clinical applications because it means, first, that the 19F NMR technique can be used in a truly non-evasive manner, and second, that it can be used to provide blood flow values in different regions of the brain.

Significance to Biomedical Research and the Program of the Institute:

There are a number of advantages to the 19F NMR approach over existing techniques for cerebral blood determination.

(1) It does not involve radioactive tracers.

(2) It is a non-invasive, i.e., it can be used to perform sequential determinations of blood flow in an individual animal.

(3) It can be used in conjunction with arterial/venous differences for oxygen and glucose, to calculate the cerebral rate of oxygen and glucose consumption.

(4) It can be used in conjunction with 31P NMR techniques that can determine ATP and PCr concentrations and the intracellular pH, and with multiple-quantum 23Na NMR techniques that can follow the transmembrane flux of sodium.

(5) It can provide spatial localization of cerebral blood flow using NMR imaging techniques.

The 19F approach is thus a good technique, in conjunction with other in vivo NMR techniques, for studying the factors that control energy metabolism. In particular, it will be useful in studying the effects of chronic administration and withdrawal of alcohol on cerebral energy metabolism in animals. The long-term goal is to apply the technique to humans to study directly the effects of alcohol on cerebral function.

Proposed Course:

(1) Previous studies in our laboratory showed that high concentration of CHF3 may affect the cerebral blood flow of different regions of the brain. We will determine the "dose response" curve of the effect of CHF3 on cerebral blood flow. These experiments will establish the highest concentration of CHF3 that does not significantly affect the cerebral blood flow.

(2) We will initiate studies on the possible short and long term toxic effects of CHF3. If no toxic effects are found, studies on humans will be initiated.

(3) We will investigate new approaches for determining arterial wash-out curves. In particular, we will determine if a mass spectrographic analysis of expired air is suitable for this purpose.

(4) We will continue our preliminary studies combining 19F NMR determination of cerebral blood flow and oxygen consumption with 31P NMR determination of cerebral bioenergetics.

Publications:

Sutton L, McLaughlin A, Dante S, Kotapka M, Sinnwell T, Mills E. Cerebral Venous Oxygen Content as a Measure of Brain Energy Metabolism with Increased Intracranial Pressure and Hyperventilation, J Neurosurgery 1990;73:927-32.

van Zijl P, Ligeti L, Sinnwell T, Alger J, Chesnick A. Moonen C, McLaughlin A. Measurement of Cerebral Blood Flow by Volume-Selective 19F NMR Spectroscopy, Magn Reson Med 1990;16:489-95.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00039-04 LMMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Cerebral Blood Flow and Energy Metabolism in the Rat

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	A. C. McLaughlin	Section Chief	LMMB, NIAAA
Others:	E. Dora	Visiting Scientist	LMMB, NIAAA
	I. Horvath	Visiting Fellow	LMMB, NIAAA

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Metabolism and Molecular Biology

SECTION

Physical Chemistry

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Rockville, Maryland 20852

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.20	1.6	0.6

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The role of humoral and neuronal factors in the control of cerebral blood flow and metabolism is controversial. We have studied the role of the adrenal/hypophysis axis and the role of sympathetic activation on cerebral blood flow and metabolism under normal and stressful conditions. Our initial work has been done with the hypercapnic model for stress, but the studies will be extended to other models for stress, e.g., hypoxia, hypoglycemia and withdrawal from chronic alcohol consumption.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description:

Investigators:

A. C. McLaughlin	Section Chief	LMMB, NIAAA
E. Dora	Visiting Scientist	LMMB, NIAAA
K. Hines	Biologist	LMMB, NIAAA
I. Horvath	Visiting Fellow	LMMB, NIAAA
T. Sinnwell	Biochemist	LMMB, NIAAA

Objectives:

To investigate humoral and neuronal factors that control the response of cerebral blood flow and metabolism to stress induced by hypercapnia, hypoglycemia, hypoxia and ethanol.

Methods Employed:

Our method for cerebral blood flow determination is a modification of the classical Kety-Schmidt technique. Catheters are inserted into the jugular veins, the femoral artery and the sagittal sinus. Systemic blood pressure, arterial PaO_2, $PaCO_2$ and pH, and body temperature are monitored. Tritiated water is infused into the jugular vein at a constant rate. During this infusion blood is withdrawn from the femoral artery and the sagittal sinus at constant rates. At the end of three minutes the animal is sacrificed with KCl, the brain exposed, and the cerebral cortex and blood samples counted for radioactivity. The cerebral blood flow is calculated from the ratio of the brain radioactivity to the arterial/venous difference in radioactivity.

Major Findings:

(1) Experiments comparing the role of different anesthetics and the role of sympathetic denervation indicate that neural mechanisms play an important role in the activation of cerebral blood flow in hypercapnia.

(2) Experiments with adrenal demodulated and adrenalectomized animals indicate that the adrenal gland plays an important role in the activation of cerebral blood flow in hypercapnia.

(3) Experiments with opiate blockers and opiate agonists indicate that part of the effect of the adrenal gland in regulating cerebral blood in hyperapnia is due to the release of opiate peptides.

Significance to Biomedical Research and the Program of the Institute:

One of the major factors that influences cerebral blood flow and metabolism is stress. However, even the mechanism of the response to the simplest model for stress (hypercapnia) is controversial. The most widely-accepted theory holds that humoral and neuronal factors play no role in the hypercapnic response. In this theory the major role is played by a local response of cerebral arteries to changes in intracellular pH. Our results show, however, that the neuronal and adrenal factors all play an important role in the hypercapnic response.

Both acute administration of alcohol and withdrawal after chronic administration of alcohol increase catecholamine levels in the urine. Also, alcohol raises plasma levels of glucocorticoids. Some of the effects of alcohol may thus be due to the induction of stress reaction. The information obtained in this project about the mechanisms underlying the stress reaction may be relevant to the effects of alcohol on cerebral blood flow and metabolism.

Proposed Course:

(1) Our second goal is to expand these studies to other stimuli that produce stress and affect cerebral blood flow and metabolism, e.g., hypoxia, hypoglycemia, seizures, etc.

(2) Our third goal is to determine if alcohol affects cerebral blood flow and metabolism by the same mechanisms that are observed with classical stress reactions.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00040-04 LMMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Electrostatic Properties of Membranes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.: A.C. McLaughlin Section Chief LMMB, NIAAA

COOPERATING UNITS *(if any)*

Physiology Department, State University of New York, Stonybrook, NY (S. McLaughlin); Biochemistry Department, University of Pennsylvania, Philadelphia, PA (J.R. Williamson).

LAB/BRANCH

Laboratory of Metabolism and Molecular Biology

SECTION

Physical Chemistry

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Rockville, MD 20852

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.9	0.2	0.7

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The surface potential of cellular membranes is an important determinant in the physiological function of the cell. We have investigated a number of factors that affect the surface potential of membranes. We have also modified the theory that has been used to calculate the surface potential, the Gouy-Chapman theory, to account for these factors.

Triphosphoinositide lipids in plasma membranes could have up to five negative charges. We previously investigated the interaction of calcium, magnesium, potassium, protons and other cations with triphosphoinositides, and determined the number of cations bound to the lipid under physiologically-relevant conditions. We also investigated proton titration curves of the lipid and found that there was a cooperative interaction between the two monoester groups in the inositol headgroup. We proposed that this interaction could have important consequences in terms of the net charge on the triphosphoinositide lipid under physiological conditions.

Project Description:

Investigators:

A. C. McLaughlin	Section Chief	LMMB, NIAAA
K. Hines	Biologist	LMMB, NIAAA
S. McLaughlin	Professor	Physiology, SUNY,
T. Sinnwell	Biochemist	LMMB, NIAAA
		Stony Brook, NY
J. R. Williamson	Professor	Biochemistry, University of PA, PA

Objectives:

This project has two objectives. The first objective is to investigate the electrostatic potential near a charged membrane, and to determine how the structure of the charged groups at the surface affect the potential. The second objective is to study the interaction of triphosphoinositide lipids with membrane bound proteins.

Methods Employed:

We used classical surface chemistry techniques to determine the zeta potential, fluorescent techniques to determine the electrostatic potential 10 A from the surface of the membrane, and NMR techniques to determine the electrostatic potential at the surface of the membrane. We also used NMR techniques to investigate the protonation of phosphomonoester groups in polyphosphoinositides, and to study the interaction of potassium with the phosphomonoester groups.

Major Findings:

The Gouy-Chapman theory predicts that if charged groups extend from the surface of the membrane, the magnitude of the electrostatic potential at the surface will be reduced, and the spatial profile of the potential will be altered. We have confirmed this prediction with sialoglycolipids (gangliosides), in which the charged sialic acid residues extend approximately 10 A from the surface.

Our previous studies indicated that, under physiologically-relevant conditions, calcium binds very weakly to gangliosides on the outer surface of cellular plasma membranes, and very weakly to triphosphoinositide lipids on the inner surface of cellular plasma membranes. The studies also suggested that under physiological conditions, potassium binds strongly to triphosphoinositides, and that there is a cooperative interaction between the two phosphomonoester groups in triphosphoinositides.

Our studies during the past year using 39K NMR to study the binding of potassium confirm that potassium ions and protons compete for binding sites on the phosphomonester groups. They also show that the cooperative interaction between the two phosphomonoester groups in the membrane system is also observed in soluble model compounds that are analogs of the polar headgroup region of the inositide lipids. This result suggests that the cooperative interaction is due to intramolecular electrostatic interactions rather than intermolecular interactions between adjacent lipids in the membrane.

Significance to Biomedical Research and the Programs of the Institute:

A number of important physiological functions of cellular membranes are dependent on the surface potential at the membrane-solution interface. This is particularly true for membrane functions that depend directly on the transmembrane potential difference, because the transmembrane potential is sensitive to changes in the surface potential. For example, it is now accepted that the effect of calcium and other divalent cations in shifting the current/voltage relationship of nerve is due to the effect of calcium on the surface potential of the nerve membrane. However, the interpretation of this effect, and many others, has been complicated by the fact that the theory that has been used to calculate the surface potential (the Gouy-Chapman theory) is obviously oversimplified, and may not be relevant to biological membranes.

In the last five years we have proposed a number of modifications to the Gouy-Chapman theory and tested them on membranes reconstituted with components isolated from biological membranes. The present project concerns two of the modifications to the theory - e.g., the effect of the detailed structure of the charged groups on the surface potential, and the effect of monovalent cation binding on the surface potential. Our results help to explain two apparent paradoxes. First, the surface potential of cells is known to be substantially smaller than the value calculated from the simple Gouy-Chapman theory, assuming the known density of charged sialic acid residues. Second, the surface potential of model phospholipid membranes containing titratable residues, i.e., the phosphomonoester groups in polyphosphoinositides, does not change significantly as the groups are titrated.

Triphosphoinositides (PIP2) play a major role in the transduction of intracellular signals (e.g., from hormones or neurotransmitters) into changes in the biochemical function of the cell. Recent work has shown that ethanol can modulate the role of PIP2 in signal transduction. The NMR techniques outlined in this project may allow us to study the physical state of PIP2 in biological membranes, and, in particular, study the interaction of PIP2 with membrane proteins.

Proposed Course:

We will extend our experiments with sialoglycolipids to sialoglycoproteins. We have developed a purification procedure for glycophorin, the major sialoglycoprotein in the erythrocyte membrane, and a procedure for the asymmetric incorporation of the protein into well-defined phospholipid vesicles. Finally, we plan to study the interaction of triphosphoinositides with glycophorin. This system might be a good model system for studying the interaction of inositol lipids with membrane proteins.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00042-03 LMMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Multiple Quantum NMR Studies of Sodium and Potassium in the Rat Brain

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	A. C. McLaughlin	Section Chief	LMMB, NIAAA
Others:	L. Ligeti	Visiting Scientist	LMMB, NIAAA
	R. Lyon	Staff Fellow	LMMB, NIAAA
	J. Pekar	Senior Staff Fellow	LMMB, NIAAA

COOPERATING UNITS *(if any)*

BEIP, NIH, Bethesda, MD (CT Moonen)

LAB/BRANCH

Laboratory of Metabolism and Molecular Biology

SECTION

Physical Chemistry

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Rockville, Maryland 20852

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.20	1.20	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We previously developed a new approach for studying trans-membrane ion gradients in the intact brain utilizing the different NMR relaxation times of intracellular and extracellular ions. Double quantum NMR spectra are more sensitive than conventional single quantum NMR spectra to changes in relaxation times. Double quantum and single quantum 23Na and 39K NMR spectra were obtained from rat brain in vivo. Upon death, the double quantum 23Na NMR signal increased by a factor of three, while the single quantum signal decreased by 20%. The results are consistent with the well-known influx of sodium ions into the cell, and suggest that double quantum sodium and potassium NMR may be useful in visualizing compromised regions of the brain.

We have extended this approach in a number of directions. First we extended the approach to study the cat brain in vivo, and the perfused rat liver. Second, we used double-quantum 23Na NMR to study compartmentation of sodium in the intact brain. Third, we combined multiple-quantum 23Na NMR with 31P NMR to study the correlation between changes in the multiple-quantum sodium NMR signals and changes in cellular energetics.

In the last year we performed a comprehensive double quantum 23 Na study of perfused liver using shift reagents. The results indicate that, in this organ, most of the double-quantum signal arises from intracellular sodium ions.

Project Description:

Investigators:

A. C. McLaughlin	Section Chief	LMMB, NIAAA
L. Ligeti	Visiting Scientist	LMMB, NIAAA
R. Lyon	Senior Staff Fellow	LMMB, NIAAA
C. T. Moonen	Director	NMR Center, NIH
J. Pekar	Senior Staff Fellow	LMMB, NIAAA

Objectives:

There are four major objectives of this project:

(1) Develop double and triple quantum NMR techniques for use with intact animals.

(2) Correlate multiple quantum NMR results with physiological results on ion transport, i.e., micro-electrode studies.

(3) Use multiple quantum NMR to study trans-membrane ion movements in physiological situations, i.e., hypoxia, focal ischemia and seizures.

(4) Combine multiple-quantum 23Na NMR approaches, which gives information on transmembrane ionic gradients with 31P NMR approaches, which give information on cellular bioenergetics.

Methods Employed:

This project employs nuclear magnetic resonance (NMR) techniques. Specially, it employs double and triple quantum NMR approaches to study the quadrupolar relaxation times of sodium and potassium ions in the brain and other organs.

Major Findings:

Our first major finding is that the kinetics of the changes in the double-quantum 23Na NMR spectrum after death vary substantially from organ to organ, but correlate with the rates of sodium influx determined by other techniques (micro-electrodes, etc.).

Our second major finding is that, in the perfused liver, most of the double quantum 23Na NMR signal arises from intracellular sodium ions. These two findings are consistent with the hypothesis that double quantum 23Na NMR can be used to follow changes in the intracellular sodium level during physiologic perturbances.

Our third major finding is that double-quantum 23Na NMR techniques can distinguish sodium ions in at least two different compartments in the brain. It is not clear at the present time if these two compartments are intracellular and extracellular sodium ions, or intracellular sodium ions in different types of cells, e.g., neurons and glial cells.

Our fourth major finding is that changes in the double-quantum 23Na NMR signal in the brain are correlated with changes in the ATP levels in the brain. This is consistent with the well established concept that the ATP level is important for the maintenance of transmembrane sodium gradients.

Significance to Biomedical Research and the Program of the Institute:

Sodium and potassium ions are inhomogenously distributed in tissues - the sodium concentration is lower inside the cell than outside, while the potassium concentration is higher inside the cell than outside. The transmembrane gradients of sodium and potassium are used to drive many physiological functions, e.g., transmission of the nervous impulse, maintenance of normal cell volume, and transport of substrates and other ions into or out of the cell.

Sodium and potassium gradients are sustained by metabolic energy and are sensitive to reductions in the cellular energy supply, or increases in the cellular activity. For example, ion-sensitive electrode measurements show that extracellular potassium levels in the brain rise in ischemia and seizures, and extracellular sodium levels fall in spreading depression.

It is difficult to extend electrode techniques to clinical situations. However, experiments are now being carried out at the In Vivo NMR Research Center at NIH on new approaches for imaging the multiple quantum sodium NMR signal. If successful, these techniques could provide a spatially resolved "map" of the transmembrane sodium distribution in the brain. The technique could then be used to provide a map of physiologically compromised areas of the brain.

A "map" of physiologically compromised regions of the brain would be of interest to many clinicians. For example, it would be useful in delineating the boundary of a focal infarction, and it might be useful in investigating the effects of chronic alcohol consumption.

Proposed Course:

There are three major avenues for future work:

(1) Application of NMR imaging techniques to localize the double-quantum 23Na signal to a well defined region in the brain.

(2) Application of the technique to study physiological perturbances in animal models: For example, we will study hypoxia, focal infarction, seizures and chronic alcohol treatment.

(3) Applications to clinical studies: If the technical problems with the technique can be overcome, preliminary studies on humans, and, finally, clinical studies will be pursued.

Publications:

Lyon R, Pehar S, Moonen C, McLaughlin A. Double-Quantum Surface Coil NMR Studies of Sodium and Potassium in the Rat Brain, Magnetic Resonance in Medicine 1991;18:80-92.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00051-02 LMMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The Effect of Ethanol on Cerebral Blood Flow and Energy Metabolism in the Rat

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	A. C. McLaughlin	Section Chief	LMMB, NIAAA
Others:	L. Ligeti	Visiting Scientist	LMMB, NIAAA
	Z. Ruttner	Visiting Fellow	LMMB, NIAAA

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Metabolism and Molecular Biology

SECTION

Physical Chemistry

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Rockville, Maryland 20852

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.4	0.4	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We developed a new technique for measuring cortical cerebral blood flow in the rat, and combined it with measurements of arterial/venous differences for oxygen and glucose to calculate cerebral oxygen consumption and cerebral glucose consumption. The major advantage of this new technique is that it can be used in the unanesthetized, freely-moving, animal. Most of the previous studies on the effect of alcohol on cerebral blood flow and metabolic rate have been performed on restrained or anesthetized animals, where it is difficult to separate the effects of stress and anesthesia from the effects of alcohol.

Project Description:

Investigators:

A. C. McLaughlin	Section Chief	LMMB, NIAAA
K. Hines	Biologist	LMMB, NIAAA
L. Ligeti	Visiting Scientist	LMMB, NIAAA
Z. Ruttner	Visiting Fellow	LMMB, NIAAA
T. Sinnwell	Biochemist	LMMB, NIAAA

Objectives:

This project has two major objectives:

(1) To develop a technique that can measure cortical cerebral blood flow in the conscious, unrestrained rat.

(2) To investigate the effects of acute and chronic alcohol intoxication and withdrawal from chronic alcohol intoxication on cerebral blood flow and metabolism.

Methods Employed:

Our method for cerebral blood flow determination is a modification of the classical Kety-Schmidt technique. Catheters are inserted into the jugular veins, the femoral artery and the sagittal sinus (confluens sinuum). Systemic blood pressure, arterial PaO_2, $PaCO_2$ and pH, and body temperature are monitored. Tritiated water is infused into the jugular vein at a constant rate. During this infusion blood is withdrawn from the femoral artery and the sagittal sinus at constant rates. At the end of three minutes the animal is sacrificed with KCl, the brain exposed, and the cerebral cortex and blood samples counted for radioactivity. Cerebral blood flow is calculated from the ratio of the brain radioactivity to the arterial/venous difference in radioactivity.

Major Findings:

(1) Under normocapnic conditions moderate blood alcohol levels (100 - 200 mg%) caused no significant effect on the cerebral blood flow, cerebral oxygen consumption, or glucose consumption. High blood alcohol levels (250 - 400 mg%) decreased all three parameters by approximately 25%. Under hypercapnic conditions high blood alcohol levels had no effect on cerebral blood flow, cerebral oxygen consumption or glucose consumption.

(2) A protocol for chronic ethanol intoxication that is compatible with the cerebral blood flow measurements has been established.

Significance to Biomedical Research and the Program of the Institute:

Acute and chronic ingestion of alcohol may depress cerebral metabolism, while withdrawal from chronic alcohol ingestion may activate cerebral metabolism.

Many of the pathological long-term effects of alcohol on the brain may be due to these effects on cerebral metabolism.

A number of studies on the effects of alcohol on cerebral metabolism have been performed in animals. Almost all of these studies have been done on anesthetized or immobilized animals. In these cases it is difficult to separate the effects of alcohol from the effects of anesthesia or immobilization stress. This is particularly important because alcohol is known to produce symptoms of a classical "stress" reaction, e.g., elevated catecholamine and corticosteroid levels, and also because anesthetics are known to effect the sympathetic system.

Proposed Course:

The studies on the acute effects of alcohol are now complete. We have devised a protocol for chronic ethanol feeding that is compatible with cerebral blood flow and metabolism studies, and in the next two years similar studies will be performed on chronic animals and animals withdrawing from chronic alcohol ingestion.

Publications:

Ligeti L, Hines K, Dora E, Sinnwell T, Huang M, McLaughlin A. Cerebral Blood and Metabolic Rate in the Conscious, Freely-Moving Rat: The Effects of Hypercapnia and Acute Ethanol Administration, Alcoholism: Clinical and Experimental Research, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AA 00052-01 LMMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

In Vivo Measurement of Intracellular Calcium in the Brain

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	A.C. McLaughlin	Section Chief	LMMB, NIAAA
Others:	L. Ligeti	Visiting Scientist	LMMB, NIAAA
	Z. Ruttner	Visiting Fellow	LMMB, NIAAA

COOPERATING UNITS *(if any)*

Bar Ilan University, Ramat Gan, Israel (A. Mayevsky)

LAB/BRANCH

Laboratory of Metabolism and Molecular Biology

SECTION

Physical Chemistry

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Rockville, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.20	0.20	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have used calcium-sensitive fluorescent dyes to measure changes in intra cellular calcium concentrations in the intact brain. Changes in intracellular calcium determined with this approach can be correlated with changes in extracellular sodium levels determined simultaneously with ion-sensitive electrodes. Preliminary results show that in hypoxia changes in extracellular sodium levels occur well before changes in intracellular calcium are observed.

Studies with perfused liver show that the dyes used for intracellular calcium determination leak out of the liver. The fluorescence can therefore not be used in the liver.

Project Description:

Investigators:

A.C. McLaughlin	Section Chief	LMMB, NIAAA
K. Hines	Chemist	LMMB, NIAAA
L. Ligeti	Visiting Scientist	LMMB, NIAAA
A. Mayevsky	Professor	Bar Ilan Univ., Israel
Z. Ruttner	Visiting Fellow	LMMB, NIAAA

Objectives:

The objectives of this project are to develop a new in vivo fluorescence approach that can be used to measure changes in the intracellular calcium levels in the brain. The method will be used first to measure changes in intracellular calcium levels during activation (e.g., seizures) and inhibition (e.g., hypoxia) of brain function. Changes in the intracellular calcium concentration will be correlated with changes in intracellular sodium levels determined by in vivo 23 Na NMR, and extracellular sodium levels using ion-sensitive electrodes. The long-term goal is to use this new fluorescence approach to follow changes in intracellular calcium during acute and chronic treatment with alcohol.

Methods Employed:

A number of studies have been published recently using calcium-sensitive fluorescent dyes to measure intracellular calcium levels in isolated cells. In these studies, the cells are washed with an esterified form of dye, which can permeate through the cell membrane. Once in the cell, the dye is cleaved by cellular proteases to form the anionic form. Since the anionic form should be impermeable to the cell membrane, it should be trapped inside the cell. Excess esterified dye is washed out of the cellular suspension, and the optical properties of the dye are used to determine the intracellular calcium concentration.

We have modified this approach so it can be used with intact brain, in vivo. A small region of the skull is removed, and the surface of the brain is superfused with an esterified form of a calcium-sensitive fluorescent dye. Excess dye is washed from the surface of the brain, and a light guide placed on the surface of the brain. The light guide is used to excite the intracellular dye, and to collect the fluorescence signals from the intracellular dye. Changes in the fluorescence signals from the dye can be used to calculate changes in the intracellular calcium concentrations.

Major Findings:

(1) Control studies in perfused liver show that in this organ the anionic form of the dye is permeable to the cell membrane, and slowly washes out of the cell. In intact brain, in vivo, the anionic form of the dye apparently does not wash out of the cell. These findings limit the in vivo use of the technique to studies with brain, and have important repercussions for the use of the technique with isolated cells.

(2) Preliminary studies indicate that in hypoxia, the extracellular sodium and potassium levels change well before any change in the intracellular calcium level is observed.

Significance to Biomedical Research and the Program of the Institute:

Many cellular functions are linked through the cytoplasmic free calcium level. The details of the coupling between the transmembrane calcium gradient and biochemical functions are, however, not clear. In the past, there have been no approaches that could be used to study free intracellular calcium concentrations. With the advent of the new approaches outlined in this project, the interrelationship between intracellular calcium levels and the biochemical functions of the cell can be studied in the intact brain in vivo.

It is known that intracellular calcium and sodium levels are closely coupled, but the details of this coupling have not been extensively investigated. The approach outlined in this project can be used in conjunction with new techniques currently being developed to study the interrelationship between changes in sodium and calcium levels in the brain.

A number of studies have suggested that the acute and chronic effects on the brain and other organs may be mediated by alterations in the intracellular calcium levels. The new approach outlined in this project will allow direct tests of these hypotheses.

Proposed Course:

First, we will complete the work on the perfused liver, to confirm that calcium-sensitive dyes can not be used with this organ. Second, we will develop an apparatus that can be used to measure both intracellular calcium (via fluorescence techniques) and extracellular sodium (via microelectrode techniques) levels in the brain. Third, we will use the newly-designed apparatus to study the response of calcium and sodium levels in the brain to hypoxia, ischemia, seizures and acute and chronic alcohol ingestion.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00053-01 LMMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

In Vivo 17O NMR Studies of Cerebral Oxygen Consumption

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	A. C. McLaughlin	Section Chief	LMMB, NIAAA
Others:	D. Fiat	Spectroscopist	LMMB, NIAAA
	L. Ligeti	Visiting Scientist	LMMB, NIAAA
	R. Lyon	Chemist	LMMB, NIAAA
	J. Pekar	Senior Staff Fellow	LMMB, NIAAA
	Z. Ruttner	Visiting Fellow	LMMB, NIAAA

COOPERATING UNITS *(if any)*

NMR Center, NIH (C. Moonen)

LAB/BRANCH

Laboratory of Metabolism and Molecular Biology

SECTION

Physical Chemistry

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Rockville, Maryland 20852

TOTAL MAN-YEARS: 0.4	PROFESSIONAL: 0.4	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project involves the development of a new non-invasive approach for imaging oxygen consumption in the brain. This new approach combines the advantages of magnetic resonance spectroscopy, which can separate signals from different metabolites in the brain, and magnetic resonance imaging, which can localize signals to well-defined regions of the brain.

If 17O-labelled oxygen is inhales, 17O NMR spectroscopy can separate and quantitate signals from 17O-labelled water, which is the end product of oxidative metabolism in the brain. The rise of this signal directly gives the rate of oxygen consumption in the brain. If this signal is imaged, the rate of oxygen consumption in different regions of the brain can be determined. At the present time, the spatial resolution of the approach is 0.8 cc.

One of the major advantages of the 17O NMR approach is that NMR techniques are non-invasive, and can be used with humans, and that 17O-labelled compounds, i.e., oxygen and water, are non-radioactive, and non-toxic.

Project Description:

Investigators:

A. C. McLaughlin	Section Chief	LMMB, NIAAA
D. Fiat	Spectroscopist	LMMB, NIAAA
L. Ligeti	Visiting Scientist	LMMB, NIAAA
R. Lyon	Chemist	LMMB, NIAAA
J. Pekar	Senior Staff Fellow	LMMB, NIAAA
Z. Ruttner	Visiting Fellow	LMMB, NIAAA
T. Sinnwell	Biochemist	LMMB, NIAAA
C. Moonen	Director	NMR Center, NIH

Objectives:

The objective of this project is to develop a new in vivo NMR approach that can be used to image oxygen consumption in the brain without the use of ionizing radioactive tracers. The long-term goal of this project is to extend the approach to clinical studies.

Methods Employed:

We utilize 170 NMR spectroscopy to follow the increase in 170-labelled water in the brain during inhalation of 170-labelled oxygen gas. We also measure the cerebral blood flow by following the wash out of a bolus injection of 170-labelled water, and we measure the recirculation of 170-labelled water in the brain from other organs by measuring the arterial concentration of 170-labelled water. All of this information is combined to calculate the cerebral rate of oxygen consumption. We obtain a three-dimensional image of the cerebral rate of oxygen consumption by imaging the 170 NMR signal from water during the bolus injection of 170-labelled water and during the inhalation of 170-labelled oxygen. At the present time the voxel size for the image is 0.8 cc>

Major Findings:

In vivo 17-O NMR imaging techniques can be used to measure the cerebral rate of oxygen consumption in 0.8 cc voxel in the cat brain.

With minor modifications the 17-O NMR technique should be non-invasive, and feasible for use with humans.

Significance to Biomedical Research and the Program of the Institute:

Spatially resolved measurements of oxygen consumption in the human brain are important in studying regional brain function in normal and localized brain disfunction in clinical situations. At the present time, positron emission tomography (PET) is the only technique capable of obtaining spatially resolved measurements of oxygen consumption in the human brain.

Although PET is a powerful approach, it suffers from a number of problems. First, PET involves high doses of radioactive tracers, which limit its use in some situations (i.e, serial studies) and excludes it from other situations (i.e., pediatric studies). Second, it is difficult to compare PET images with images taken with different techniques (i.e., MRI or CT) to accurately assign functional parameters to different anatomical regions of the brain.

NMR spectroscopic imaging techniques do not involve radioactive tracers, and can be easily combined with MRI techniques to provide a direct comparison between functional and anatomical parameters. 17O-spectroscopic imaging techniques for measurement of oxygen consumption thus have an important potential for neurological and clinical studies.

Chronic ethanol consumption causes serious neurological deficits. Non-invasive clinical approaches for the study of oxygen consumption in different regions of the brain would provide a technique both for studying the pathophysiology of these effects, and for monitoring therapeutic approaches.

Proposed Course:

The 17-O NMR approach for measuring oxygen consumption in the brain has been shown to be feasible in cats. Further work is needed before the technique can be applied to humans. First, the approach uses and invasive arterial injection of 17O-labelled water to measure cerebral blood flow. To overcome this problem, we will develop 19F NMR imaging approaches using inhalation of CHF3 to measure cerebral blood flow. Second, the approach uses surface coils, which are useful for monitoring regions of the brain near the surface, but cannot be used to obtain images of the entire brain. To overcome this problem we will build specialized "whole-head" coils tuned for 17O NMR. Third, the approach uses invasive arterial cannulation to obtain the time-dependence of the arterial concentration of 17O-labelled water. To overcome this problem, we will utilize the fact that the concentration of 17O-labelled water in the blood does not change during passage through the lungs, and explore approaches which utilize venous cannulation.

Publications:

McLaughlin AC, Pekar J, Ligeti L, Ruttner Z, Lyon R, Sinnwell T, van Gelderen, P, Fiat D, Moonen CTW. In Vivo Measurement of Cerebral Blood Flow and Oxygen Consumption Using 17O Magnetic Resonance Imaging. In: Zachery S, Witt E, eds. Imaging in Alcohol Research, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00056-01 LMMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

In Vivo NMR Exercise Studies in Panic Disorder and Alcoholic Patients

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	A. C. McLaughlin	Section Chief	LMMB, NIAAA
Others:	T. Sinnwell	Chemist	LMMB, NIAAA
	D. George	Acting Section Chief	LCS, NIAAA
	M. Linnoila	Scientific Director	NIAAA

COOPERATING UNITS *(if any)*

Diagnostic Radiology Department, Clinical Center, NIH (J. Frank)

LAB/BRANCH

Laboratory of Metabolism and Molecular Biology

SECTION

Physical Chemistry

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Rockville, Maryland 20852

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.4	0.4	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

31P NMR techniques have been developed for studying metabolism in skeletal muscle during and after defined exercise protocols. The techniques have been tested on control subjects, and in the next year will be used to study panic disorder and alcoholic patients. This study should provide information on physiological and metabolic disorders in these patients.

Project Description:

Investigators:

A. C. McLaughlin	Section Chief	LMMB, NIAAA
J. Frank	Chief	DRD, CC
T. George	Chief, Clinical Studies	LCS, NIAAA
M. Linnoila	Scientific Director	NIAAA
T. Sinnwell	Chemist	LMMB, NIAAA

Objectives:

(1) To develop instrumentation for exercise studies in the clinical magnet at the In Vivo NMR Center, and to modify the clinical NMR system at the In Vivo NMR Center so that it can perform exercise studies.

(2) To obtain time-resolved 31P NMR spectra from gastrocnemius muscle of normal subjects, panic disorder patients, and alcoholic patients during control, exercise and recovery periods.

(3) To compare the ATP, inorganic phosphate and phosphocreatine levels, and the intracellular pH, during control, exercise and recovery periods.

(4) To correlate biochemical changes measured in exercising gastrocnemius muscle by 31P NMR with clinical symptoms in the two patient groups.

(5) To determine if NMR exercise studies can elucidate underlying metabolic disorders in panic disorder and alcoholic patients.

Methods Employed:

We use 31P NMR spectroscopy to determine the concentrations of phosphorylated metabolites in the human gastrocnemius muscle. The subject lies in the clinical NMR magnet in a prone position, with the calf on a surface coil, and the foot in a pedal attached to a weight. 31P NMR spectra are acquired approximately every minute while the subject performs repeated plantar flexions. The weight load will be adjusted to the size of the gastrocnemius muscle in the individual patients.

Major Findings:

(1) Exercise equipment has been designed, constructed and installed in the clinical NMR system, and the software and hardware on the clinical NMR system has been redesigned to allow sequential acquisition of 31P NMR spectra during exercise protocols. The exercise equipment and the redesigned software and hardware have been tested with exercise studies on control subjects, and exceed the specification necessary for clinical protocols.

(2) Preliminary exercise studies with control subjects have been used to define a standard exercise protocol that will be used for clinical studies. Normal subjects have a moderate decrease in phosphocreatine levels, a moderate

increase in inorganic phosphate levels, but no alterations in intracellular pH during the exercise protocol.

Significance to Biomedical Research and the Program of the Institute:

The rate constant for recovery of Phosphocreatine and inorganic phosphate levels after cessation of exercise is related to the rate of energy production in muscle. Previous 31P NMR studies have shown that patients with reduced energy production rates due to mitochondrial myopathies, glycolytic defects, peripheral vascular disease or chronic heart failure generally have substantially (i.e., up to an order of magnitude) reduced rates of recovery of phosphocreatine and inorganic phosphate after exercise.

If the rate of oxidative metabolism in the muscle cell can not maintain an increased work lead, the rate of glycolysis may increase. Under these conditions the intracellular pH will drop. Previous 31P NMR studies have shown that with many patients a moderate exercise protocol can produce substantial changes in muscle pH under conditions where only minimal changes in the muscle pH would be observed in control subjects.

The significance of the proposed studies lies in the fact that 31P NMR is the first, and at this point the only, non-invasive technique that can be used to monitor biochemical and physiological function in intact muscle. This functional information can be correlated with clinical information to investigate the biochemical and physiological derangements that give rise to specific pathological symptoms. Many muscular disorders would be amenable to this approach, and one of the long-term objectives of this proposal is to provide the NIH/ADAMHA community with established 31P NMR techniques that can be adapted to the individual clinical programs. At the present time, NINDS, NIAMS and NIDR are involved in exercise studies using the instrumentation and techniques developed in this project.

Panic Disorder syndrome has been shown to have a relationship to chronic alcoholism. In both panic disorder syndrome and chronic alcoholism muscle energy metabolism may be impaired. The techniques outlined in this project will allow clinical studies of muscle metabolism in panic disorder patients and chronic alcoholic patients, and may provide a basis for diagnostic and treatment of the myopathic complications of these diseases.

Proposed Course:

(1) To complete the technical development of the exercise equipment and the modifications of the clinical NMR System to facilitate clinical exercise studies.

(2)To complete the development of the exercise protocol and collect data on normal controls.

(3) To begin clinical exercise studies on panic disorder patients and chronic alcoholic patients.

Publications:

None

Annual Report of the
Laboratory of Physiologic and Pharmacologic Studies
Division of Intramural Clinical and Biological Research
National Institute on Alcohol Abuse and Alcoholism
October 1, 1990 to September 30, 1991
George Kunos, M.D., Ph.D., Chief

Introduction

Fiscal Year 1991 has been a very productive year for the Laboratory of Physiologic and Pharmacologic Studies (LPPS). Scientists in the LPPS have built on important breakthroughs achieved in previous years as well as initiated potentially important new lines of research. As a general feature, new information has continued to emerge from parallel electrophysiological, biochemical, neurophysiological, and molecular biological studies, which illustrates the mutually beneficial interactions among our scientists with different background and interest.

Work in the LPPS has had a key role in establishing certain neurotransmitter receptors as major and specific targets for the neurotoxic effects of ethanol. The potential role of the NMDA-subtype of glutamate receptor in mediating the neural and cognitive impairments caused by ethanol has been first suggested by electrophysiological and biochemical studies conducted in the LPPS. Current work has explored and identified possible underlying mechanisms. Another major neurotransmitter system whose role in the actions of ethanol has been under intense scrutiny in the LPPS is GABA and GABA-gated chloride channels. Electrophysiological studies in cultured cells have indicated that ethanol potentiates the GABA-gated chloride current in some but not in other neurons. Neurophysiological studies in anesthetized animals have established that the well known effect of ethanol in inhibiting the cardiovascular baroreceptor reflex is related to a potentiation of the baroreflex inhibitory action of endogenous GABA at a well defined site in the lower brainstem. This highly localized interaction appears to involve both GABA-A and GABA-B receptors. Recent work has highlighted the potential role of yet another neurotransmitter receptor, the 5-HT3 receptor, in the actions of ethanol. In cultured neurons ethanol was found to potentiate the ion current gated by the 5-HT3 type of serotonin receptor. In behavioral studies 5-HT3 antagonists were found to inhibit the discriminative stimulus effects of ethanol. In addition to important progress in the above studies directly related to the biology of ethanol, there have been important developments in other areas of research in the LPPS, such as the cloning of the gene for a novel, testis-specific form of calcineurin, or the cloning of a fat cell specific beta-receptor that might be related to obesity in genetically obese rats. These are just some of the highlights of current work in the LPPS, and important developments in other research projects are detailed below.

During the current fiscal year Dr. Paula Hoffman, Chief of the Section on Receptor Mechanisms, has left the LPPS to take up a position as Professor of Pharmacology at the University of Colorado at Denver. Dr. Hoffman has been an important member of our Institute and the LPPS who has made major contributions to our research efforts and whose presence and contributions will be missed.

Scientific activities in the LPPS have included a very active and well attended seminar program with some prominent speakers. The list includes Prof. Lev

Bergelsen (Moscow), Dr. Harold Kalant (Toronto), Dr. Enoch Gordis (NIAAA), Dr. William Lands (NIAAA), Dr. George Uhl (NIDA), Dr. M.K. Ticku (San Antonio), Dr. Hreday Sapru (Newark), Dr. David Goldman (NIAAA), Dr. Ken Lukowiak (Calgary) and Dr. Peter Smith (Calgary).

1. Office of the Chief

Areas of ongoing research include brainstem neuronal mechanisms in cardiovascular regulation and in the cardiovascular effects of ethanol, mechanism of the inverse regulation of alpha1- and beta2-adrenergic receptors, interactions between the immune system and adrenergic receptors, mechanisms of T cell maturation and the neurobiology of cytokines, and the effects of ethanol on GABA-gated chloride-fluxes.

A. Brainstem Neural Mechanisms in Cardiovascular Regulation

Ethanol has well documented hypertensive and pressor effects. Among the possible underlying mechanisms, inhibition of the depressor baroreflex response has been well characterized in a rat model. Since ethanol is known to potentiate some GABA-A receptor mediated responses and the baroreflex is under GABA-ergic inhibitory control at the level of its first synapse in the dorsal medullary vagal complex (DVC), we tested the hypothesis that ethanol inhibits the baroreflex by potentiating the inhibitory action of endogenous GABA at the DVC. The current studies are an extension of earlier, preliminary findings and have been done in urethane-anesthetized rats. Ethanol, 1 g/kg iv. or 25-200 nmol microinjected bilaterally into the DVC inhibited baroreflex bradycardia elicited by the iv injection of the pressor agent, phenylephrine, but not the bradycardic response to vagal stimulation. The baroreflex inhibitory effect of ethanol was abolished by depleting endogenous GABA through pretreatment of rats with 3-mercaptopropionate. Ethanol potentiated the pressor and tachycardic responses to intra-DVC microinjections of the GABA-A agonist, muscimol and, to a lesser degree, the GABA-B agonist, baclofen. Microinjection of the selective GABA-A antagonist, bicuculline, or the selective GABA-B antagonist, 2-hydroxy-saclofen, significantly reduced the baroreflex inhibitory action of ethanol. Although 2-OH-saclofen did not influence the effects of muscimol, it did prevent the potentiation of the effects of muscimol by ethanol. These results indicate that ethanol inhibits baroreflex bradycardia by potentiating the similar effects of endogenous GABA in the DVC, and that both GABA-A and GABA-B receptors are involved in this effect.

B. Interaction Between the Immune System and Adrenergic Receptors

Our earlier studies demonstrated that interleukin-1 (IL1) upregulates beta-adrenergic receptors (BAR) in cultured human lung tumor cells. In the current fiscal year we examined the selectivity of this regulation for BAR subtypes and its mechanism at the level of the expression of the receptor genes. The density and affinity of beta1 (B1AR) and beta2-receptors (B2AR) in A549 cells were examined by computerized curve fitting of radioligand (125I-pindolol) binding and its suppression by subtype selective antagonists. Steady state levels of B1AR and B2AR mRNAs were quantified by DNA excess solution hybridization assays, using probes designed and synthesized in our laboratory. The expression of BAR in A549 cells is cell density dependent: preconfluent cultures have more receptors than confluent ones. The difference was found to be due to loss of B2AR in confluent cells, while the density of B1AR did not change. IL1-alpha (IL1a) did not influence BAR densities in preconfluent cells, but significantly and selectively increased B2AR density in confluent cells, without affecting the affinity of B2AR. The effect of IL1a in confluent cells was potently antagonized by a recombinant antagonist against typeI IL1 receptors. The increase in B2AR density by IL1a was preceded by an increase in the levels of B2AR mRNA, while B1AR mRNA was unaffected. This increase in B2AR mRNA was related to an increase in the rate of its transcription, as evidenced by nuclear run-off assays. These findings indicate that IL1a acting on typeI IL1 receptors in A549 cells causes a cell-density-dependent, selective upregulation of B2AR through increased transcription of the B2AR gene.

C. Inverse Regulation of Alpha1- and Beta2-Receptors

Earlier studies have established that hypothyroidism in rats leads to a conversion of the adrenergic activation of liver glycogenolysis from an alpha1b- (A1bAR) to a beta2-receptor (B2AR)-mediated event, while the relative dominance of the two receptors shows an opposite change (beta to alpha1) in the heart of the hypothyroid animal. We have quantified mRNAs for A1bAR, B1AR and B2AR by DNA excess solution hybridization assays in the heart, lung, and liver of euthyroid and hypothyroid (TX) rats and hypothyroid rats treated with a single dose of T4 (TX+T4). Thyroidectomy decreased B1AR and B2AR mRNAs in heart and lungs and A1bAR mRNA in liver, whereas B2AR mRNA in liver and A1bAR mRNA in lung and heart were significantly increased. These changes were reversed in tissues from TX+T4 rats. This is the first demonstration that thyroid state regulates adrenergic receptor mRNA levels, and that this regulation is tissue- and receptor-specific. These changes correlate with the well documented changes in the cellular densities and physiological reactivities of adrenergic receptors in different tissues. Dissociation of euthyroid rat hepatocytes leads to a rapid (<4 hr), in vitro change from B2AR- to A1bAR-mediated glycogenolysis similar to that observed in vivo in the liver of hypothyroid animals. We now find that A1bAR mRNA is decreased and B2AR mRNA is increased significantly as early as 2 hours after dissociation of the cells, which is probably the mechanism underlying the subsequent conversion of the adrenergic receptor response.

D. Mechanisms of T cell Maturation and the Neurobiology of Cytokines.

Loss of T lymphocytes is key to the pathomechanism of AIDS. Therefore, finding ways to increase T cell production in the thymus may have therapeutic potential. A monoclonal antibody generated against immature T cell blasts was found to have costimulatory effect on the proliferation of immature T lymphocytes. The antigen recognized by this antibody was identified as the dipeptidyl peptidase, DPPIV, a membrane proteolytic enzyme. This observation opens new possibilities in understanding and influencing the development of T cells.

The inflammatory cytokine, interleukin-1 (IL-1), induces HIV transcription in macrophages. HIV-related dementia is related to the presence of HIV-infected macrophages in the brain. A study of the expression of inflammatory cytokine (IL-1, TNF) genes in the mouse brain has indicated that under basal conditions these genes are expressed at a very low level, detectable only by PCR analysis. However, systemic administration of known inducers of inflammatory cytokine production results in a marked increase in the expression of the cytokine genes in various brain regions. These findings suggest that inflammatory processes, such as those associated with AIDS infection, can induce cytokine production in the brain. Therefore, it is possible that antiinflammatory treatment of AIDS patients may slow the development of AIDS-related dementia.

E. Effects of Ethanol on GABA-gated Chloride Fluxes.

Evidence from biochemical, behavioral, genetic, and some electrophysiological studies indicate that certain biological effects of ethanol may be mediated by potentiation of the action of GABA at GABA-A receptors, although there are also findings, mostly electrophysiological, that do not support this notion. To examine this question by a novel approach, chloride fluxes in cultured neurons have been measured by digital microscopic fluorescence imaging using the chloride-sensitive intracellular fluorescent probe, SPQ. The rate of chloride efflux from the cells down an imposed concentration gradient was significantly accelerated by GABA, and this effect of GABA could be antagonized by the GABA-A antagonist, bicuculline. Preliminary observations also indicate that physiologically relevant concentrations of ethanol further accelerate the GABA-induced increase in chloride efflux. This experimental model and methodology is being used to further analyze the factors involved in modulating the effects of GABA at GABA-A receptors, such as cAMP-dependent mechanisms, ethanol and, possibly, interactions with GABA-B receptors.

2. Section of Electrophysiology

The research program of the Section of Electrophysiology is directed toward elucidating the molecular and cellular basis of alcohol's actions in the nervous system. The behavioral effects of alcohol are well known; however, the mechanisms by which alcohol produces those effects have not been established. The investigations in the Section use primarily electrophysiological methods such as intracellular recording, voltage-clamp and patch-clamp techniques to study the actions of alcohol on mammalian neurons and neuronal membrane ion channels. For organizational purposes the research program of the Section can be divided into two general areas: intrinsic nerve cell excitability and synaptic mechanisms.

A. Intrinsic nerve cell excitability and voltage-gated ion channels:

Previous investigations have shown that the administration of ethanol can alter the firing rate of neurons in the central nervous system, suggesting that ethanol may affect the intrinsic excitability mechanisms of central neurons. Recent biophysical and molecular biological studies have demonstrated that voltage-gated ion channels underlie the intrinsic excitability of mammalian neurons. In the Section of Electrophysiology, we have identified and characterized voltage-gated ion channels in adult mammalian neurons. Three types of voltage-gated ion channels have been characterized: sodium channels, calcium channels and potassium channels. Of these, two types of sodium currents have been identified, a low-threshold, fast sodium current that is tetrodotoxin (TTX)-sensitive and high-threshold, slower sodium current that is TTX-resistant. For calcium currents, two types have been studied, a low-threshold transient calcium current and a high-threshold sustained calcium current. In addition, three types of potassium currents have been characterized, a low-threshold transient potassium current that is sensitive to 4-aminopyridine (4-AP), a high-threshold sustained potassium current that is sensitive to tetraethylammonium (TEA) and a sustained calcium-activated potassium current. We have studied the effect of ethanol on the ion currents associated with these voltage-gated ion channels and found that pharmacologically relevant concentrations of ethanol (viz. 100 mM or less) have little or no effect on these channels.

B. Synaptic mechanisms and transmitter-gated ion channels:

The excitability of nerve cells is also regulated by the action of neurotransmitters. Recent studies on the identification of neurotransmitters in the brain indicate that the major excitatory and inhibitory neurotransmitters are glutamate and gamma-aminobutyric acid (GABA), respectively. In the Section of Electrophysiology, we have investigated the effect of ethanol on glutamate- and GABA-gated ion channels in mammalian neurons. The ion current activated by the glutamate receptor agonist N-methyl-D-aspartate (NMDA) was inhibited by ethanol. The inhibition increased in a concentration-dependent manner over the concentration range 5 to 50 mM, a range that produces intoxication. By contrast, this concentration range of ethanol had relatively little effect on the current activated by the glutamate receptor agonists kainate and quisqualate. The ethanol-induced inhibition of NMDA-activated current was not use- or voltage-dependent and did not appear to involve regulatory sites on the NMDA channel, but was correlated with the hydrophobicity of different alcohols, suggesting a novel interaction with a hydrophobic region of the NMDA channel. In addition, the potency for inhibition of the NMDA current by several alcohols was linearly related to their intoxicating potency, suggesting that alcohol-induced inhibition of responses to NMDA receptor activation may contribute to the neural and cognitive impairments associated with intoxication.

Ethanol also inhibited kainate- and quisqualate-activated currents. At ethanol concentrations greater than 50 mM, ethanol increasingly inhibited kainate- and quisqualate-activated currents in a concentration-dependent manner, with 200 mM ethanol producing a 45-60% inhibition of these currents. Over this concentration range, ethanol produces increasing depression of central nervous system excitability ranging from sedation to coma and death from central respiratory depression. Since kainate and quisqualate channels are known to mediate the majority of fast EPSPs (excitatory postsynaptic potentials) in the central nervous system (CNS), this suggests that the ethanol-induced inhibition of kainate- and quisqualate-activated current may contribute to the general anesthetic actions of ethanol. This is supported by the observation that general anesthetics such as pentobarbital and trichloroethanol (the activate metabolite of chloral hydrate) potently inhibit kainate- and quisqualate-activated currents.

The effect of ethanol on membrane ion current activated by the inhibitory neurotransmitter GABA was also studied. In contrast to glutamate-gated ion channels, where ethanol inhibited the ion current in virtually all neurons studied, ethanol had no effect on GABA-activated Cl^- current in some neurons and potentiated GABA-activated Cl^- current in other neurons. This differential sensitivity may result from $GABA_A$ channel subunits which confer ethanol sensitivity on the channel or from regulation of the ion channel by processes such as phosphorylation or glycosylation. GABA-activated Cl^- current was also potentiated by benzodiazepines, suggesting that augmentation of this current by ethanol may contribute to the anxiolytic actions of ethanol.

In view of the sensitivity of glutamate- and GABA-gated ion channels to ethanol, the Section of Electrophysiology has also recently begun investigating the effect of ethanol on other transmitter-gated ion channels. Ethanol was found to potentiate the ion current associated with the serotonin-activated 5-HT_3 type of transmitter-gated ion channel. The potentiation of 5-HT_3 current was concentration-dependent over the concentration range 25 to 100 mM. Behavioral experiments suggest that this effect may be associated with the recognition of ethanol action. We have also recently found that ethanol inhibits the ion current associated with ATP-gated ion channels. The inhibition is concentration-dependent over the concentration range 6 to 250 mM. The functional significance of ethanol inhibition of ATP-activated ion current remains to be determined, since the function of ATP-gated ion channels in the CNS is not yet known.

The observations suggest that neurotransmitter-gated ion channels may be molecular sites of ethanol action in the CNS. Moreover, the behavioral effects of ethanol may result from the actions of ethanol on these receptor-gated channels.

3. Section on Molecular Neurobiology

Neurotransmitter receptor systems are essential in all cellular communication and are the targets for therapeutic intervention in a large number of diseases. Research in the Section on Molecular Neurobiology is directed towards the study of neurotransmitter receptor structure and regulation and is currently focused on three major areas:

A. Elucidation of the relationship between neurotransmitter receptor structure and function using molecular biological and biochemical techniques. This work involves gene cloning, site-directed-mutagenesis and gene expression in cultured cells and Xenopus oocytes. The molecular properties of two major classes of membrane receptors are being investigated: G protein-coupled receptors, which mediate their cellular actions through guanine nucleotide binding proteins, and $GABA_A$ receptors, which function as ligand-gated ion channels and have been implicated as a specific site of alcohol's actions in the central nervous system. Current studies are aimed at identifying receptor domains involved in ligand binding, agonist activation and receptor down regulation.

Studies during the past year have demonstrated that conserved cysteine residues in G protein-coupled receptors are critical for normal receptor function. Furthermore, not only to muscarinic agonists but also to beta-adrenergic agonists (receptor cross-talk). Because these two types of receptors are often expressed in the same cell, these findings have important implications in our understanding cellular responsiveness following long-term agonist exposure.

We have also begun studies to examine the structure/function relationship of GABA receptors. Glycosylation of GABA receptors expressed in Xenopus oocytes was shown to be required for the expression of a functional receptor-channel complex. The role of each of the putative sites for glycosylation on GABA receptor subunits is currently under investigation. Furthermore, the site(s) within the receptor responsible for high affinity benzodiazepine binding are being mapped using in vitro mutagenesis techniques.

We have also identified a putative new beta-adrenergic (B_{3A}) receptor subtype that is expressed in adipose tissue. This receptor represents the predominant beta-adrenergic receptor in fat cells and its expression is markedly reduced in genetic models of obesity, suggesting that it may play a role in the development of this disorder.

B. Understanding the molecular mechanisms involved in regulation of receptor expression. The regulatory regions of neurotransmitter receptor genes that control gene transcription and its modulation by various cellular factors as well as tissue-specific gene expression are being studied. PCR mapping of the DNA sequences upstream of the coding region of the beta1 subunit of the GABA receptor gene has revealed two distinct non-coding exons that appear to be utilized in human brain. Ongoing studies aim to identify the tissue distribution of these different exons and their effects on the expression of the beta1 subunit protein.

C. Identification of DNA polymorphisms in neurotransmitter receptors genes. Through collaborative studies with other investigators within NIAAA, the Section is involved in identifying informative polymorphisms within genes encoding neurotransmitter receptors and utilizing these markers to test for genetic linkage in a number of neurological disorders including alcoholism. One of the introns of the GABA beta1 subunit gene was found to contain a tetranucleotide repeat sequence (GATA). Amplification of this region with PCR revealed the presence of 7 alleles and a high degree of polymorphism in human populations. This polymorphism can now be used to screen for any linkage between the beta1 gene and known genetic disorders. These studies are being extended to include characterization of the genomic structure of the alpha6 and gamma2 subunits of the GABA receptor.

4. Immunology Section

The Section on Immunology conducts research to examine the immunological and neurobiological consequences of alcohol abuse and alcoholism (i.e., intoxication, dependence and withdrawal) and disorders of the immune system, such as AIDS. Since these pathologies may reflect the impairment or alteration of normal signal transduction events, investigations are being carried out on the fundamental mechanisms by which lymphoid and neural cells respond to effectors. Research focuses on the role of cyclic nucleotide and phosphoprotein metabolism in such " signaling pathways " and the regulation of these by Ca^{2+} and calmodulin. The Section emphasizes the use of newer molecular biological approaches, in concert with classical biochemical and immunological methods, to characterize these systems. The following summary provides a brief overview of our current work and future plans and is divided into three main areas.

A. Molecular biology of calmodulin-dependent signal transduction pathways

Intracellular signaling events mediated by cAMP are important in modulating the biochemical response of excitable cells. Ultrastructural studies with calmodulin-dependent cyclic nucleotide phosphodiesterase have revealed a pattern of intra-dendritic compartmentalization that localizes this enzyme to regions apposed to asymmetric synapses, with little staining of other areas of the dendritic shaft. Such a finding, along with previous work that demonstrated transsynaptic regulation of expression, strongly supports a direct role for cyclic nucleotides in Ca^{2+}-dependent regulation of post-synaptic membrane potential, in addition to the effect on phosphorylation cascades mediated by cytosolic protein kinases.

Our prior work had demonstrated multiple molecular forms of the calmodulin-dependent protein phosphatase, calcineurin, in mammalian tissues. This complexity derived from two distinct genes for the catalytic subunit of this enzyme, each of which produced additional isoforms through alternative splicing. We have now cloned a third mammalian gene that appears to be a non-neural, testis-specific form. This form may be important in sperm motility, since others have reported a calmodulin-stimulated phosphatase activity that is intrinsically associated with sperm flagella. Future studies employing transgenic animals will assess the functional role of this enzyme in reproduction. We have extended our examination of evolutionary and structural relationships by cloning the gene from *Aspergillus nidulans*, which shows conservation of the intron/exon boundaries found in the mammalian genes. In addition, we have studied the regulation of gene expression in *Neurospora crassa* and *Dictyostelium discoideum*, where this enzyme appears to play a role in morphogenic and developmental events. These systems will be used for gene disruption experiments to determine the effect of mutation on phenotype. Ongoing studies using a chimeric proteins containing a recombinant fungal catalytic domain fused to variable regions of the mammalian genes are designed to identify the structural elements contributing to differences in phosphoprotein substrate specificity of this enzyme.

A potentially important, neuron-specific protein that interacts with calmodulin (NCAP-36) has been characterized, displaying an unusual anatomical distribution. Phosphorylated isoforms of the protein exist *in vivo* and this modification can be altered by calmodulin. Protein sequencing data show no similarities to other enzymes, indicating that it is a novel protein. Future molecular cloning studies will be used to investigate its physiologic role.

B. Modulation of phosphoprotein metabolism during signaling events.

Control of protein phosphorylation is a well-established mechanism for regulating intermediary metabolism, cytoskeletal organization and receptor function. Our ongoing collaborative work, using primary neuronal cultures, implicates the protein phosphatase, calcineurin, in the regulation of support structures that are needed for neurite outgrowth. In order to further these findings, we are establishing additional culture systems, including models for neuronal development in microorganisms, to assess the potential regulation by protein phosphatases of cytoskeletal proteins involved in morphogenic changes.
Calmodulin-dependent dephosphorylation appears to be a general mechanism for "down-regulation" of cAMP response systems; important substrates for cAMP-dependent protein kinases such as DARPP-32, phosphorylase kinase, and the ß-subunit of the L-type Ca^{2+} channel are among the best substrates known for calcineurin. To examine the potential interaction between this phosphoprotein phosphatase and its substrates, we have developed anti-peptide antibodies against different phosphatase isoforms and against various potential substrate sites to examine their co-localization and to modify cellular responses. We have also cloned and expressed several cAMP-regulated phosphoproteins in brain and will be evaluating potential area of "cross-talk" between these systems using molecular reagents such as anti-sense oligonucleotides.

We have also initiated a new experimental strategy to identify the interacting components in lymphocyte signaling pathways through analysis of two-dimensional gel electrophoresis phosphorylation patterns. By carefully determining the corresponding protein kinases and phosphatases that regulate changes in specific phosphoproteins, we hope to develop a "fingerprint" of interactions that may be singular for different cell types or effector-induced responses. Such a overall pattern may allow for the detection of altered "signaling competency" states in individuals that are immunocompromised, such as in alcoholism or immune diseases such as AIDS.

C. Involvement of signaling events in alcoholism and pathologies affecting the nervous and immune systems.

The participation of signal transduction in the etiology of diseases such as alcoholism and AIDS has not been examined in detail. However, disturbances in the "processing" of information could be involved, causally or as reflected by symptoms, in the underlying basis of these diseases. We have initiated a broad effort to examine aspects of phosphoprotein and cyclic nucleotide metabolism related to these specific pathologies. Using well-characterized murine models for alcoholism, we have compared the mRNA expression of calcineurin in normal and treated animals that have acute or long-term behavioral responses (e.g., withdrawals, seizures); to date, no apparent changes have been observed.

The effects on human lymphocytes of exposure to HIV coat proteins are being examined by analysis of phosphoprotein metabolism under unstimulated and immunomodulated conditions (see the electrophoresis strategy above). We are also investigating the changes in phosphoprotein profile that may be linked to early events in T-cell activation or after exposure to HIV coat proteins. These studies also may provide useful information regarding the involvement of signaling events in the pathology of other disease states. We anticipate future studies in which inbred strains of mice that exhibit stereotyped neuropathologies are surveyed in order to assess differences or abnormalities in the Ca^{2+}-regulated signaling pathways studied in this group.

5. Section on Receptor Mechanisms

With the departure of its Chief and some of its members, this has been the last year of operation of the Section on Receptor Mechanisms as an organizational unit. In general, research in the Section has been directed toward understanding the neurochemical mechanisms responsible for neuroadaptive responses to ethanol, such as functional tolerance and physical dependence, with special emphasis on the role of neurotransmitter receptors and receptor/effector coupling. Another major focus was the role of vasopressin in maintaining functional tolerance to ethanol.

Earlier work in the Section indicated that in certain brain regions chronic ethanol treatment decreases agonist stimulation of adenylate cyclase, suggesting an effect on Gs proteins or Gs protein function. Western blot analyses of two forms of Gs alpha did not reveal any quantitative difference after ethanol treatment, suggesting that the properties rather than the amount of G proteins may be affected.

The mechanism of ethanol-inhibition of the NMDA receptor was examined in primary cultured cerebellar neurons. Ethanol inhibition of NMDA-induced calcium fluxes was additive to that produced by PCP or magnesium, indicating that ethanol does not act within the NMDA channel. The lack of effect of ethanol on NMDA binding affinity further suggest that the interaction is not with the receptor binding site. The finding that high concentrations of glycine as well as other agonists at the glycine site were able to reverse ethanol-inhibition of the NMDA-response suggest that ethanol interferes with the concerted action of glutamate and glycine at the NMDA receptor. Chronic ethanol treatment also affects the NMDA receptor system, these receptors being upregulated in hippocampus and some other brain regions. It was also found that the ethanol-induced upregulation of NMDA receptors parallelled susceptibility to ethanol withdrawal seizures, which suggests a role for the NMDA-gated ion channel in the effects of ethanol withdrawal.

The demonstrated role of vasopressin (AVP) in the maintenance of ethanol tolerance prompted studies of the effects of ethanol on the synthesis of AVP in the brain. Chronic ethanol exposure was found to decrease hypothalamic AVP mRNA in both rats and mice. These results indicate that an increase in AVP synthesis is not necessary for the hormone to maintain functional tolerance.

A low fluoride-stimulated platelet adenylate cyclase activity was found to effectively discriminate alcoholic and non-alcoholic individuals, thus fulfilling the criterion for a 'marker'. Current studies have found familial transmission of this trait, with the major gene being transmitted as a Mendelian co-dominant.

Finally, electrophysiological and behavioral evidence implicating the serotonin 5-HT3 receptor in some effects of ethanol prompted a study of the effects of ethanol on 5-HT3 receptor binding. Ethanol (12.5-100 mM) was found not to affect either agonist or antagonist binding to the 5-HT3 receptor in NCB20 somatic cell hybrids, which suggests that the site of ethanol's action may instead be at the level of receptor-effector coupling.

6. Unit on Special Projects

The Unit's area of research is the behavioral pharmacology of ethanol. Studies in the current year have been aimed at clarifying the role of certain neurotransmitter systems (GABA, glutamate, serotonin) in the discriminative stimulus effects of ethanol. It was found that in both pigeons and rats the discriminative stimulus effects of ethanol could be inhibited by 5-HT3 receptor antagonists. Furthermore, the discriminative stimulus effects of NMDA receptor antagonists and of GABA-A receptor agonists were similar to those of ethanol. In a separate study, the role of vasopressin in learning vs retaining information was analyzed by comparing the performance of normal rats and rats with diabetes insipidus using a T-maze alternation task. The results support the role of vasopressin in the retention but not the acquisition of information.

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Laboratory of Physiologic and Pharmacologic Studies
October 1, 1990 to September 30, 1991

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00401-04 LPPS

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Interaction Between the Immune System and Adrenergic Receptors

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	G. Kunos	Chief	LPPS, NIAAA
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	T. Nakane	Visiting Fellow	LPPS, NIAAA
	T. Szentendrei	Visiting Fellow	LPPS, NIAAA
	L. Takacs	Visiting Associate	LPPS, NIAAA
	M. Virmani	Research Chemist	LPPS, NIAAA

COOPERATING UNITS *(if any)*

SUNY, Stonybrook, NY (C.C. Malbon)

LAB/BRANCH

Laboratory of Physiologic and Pharmacologic Studies

SECTION

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CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The regulation of beta1 and beta2-adrenergic receptors (beta1AR and beta2AR) and receptor gene expression by interleukin-1alpha (IL-1a) was studied in cultured A549 human lung adenocarcinoma cells. The density and affinity of beta1AR and beta2AR were analyzed by computerized curve fitting of 125-I-pindolol binding and its displacement by subtype selective antagonists. Steady state levels of receptor mRNAs were quantified by DNA excess solution hybridization assays. A549 cells in preconfluent cultures had fewer beta1AR than beta2AR (beta1: 1.9±0.3 vs beta2: 4.0±0.5 fmol/mg protein, mean ± SE), but lost most of their beta2AR upon reaching confluency (beta1: 2.7±0.4, beta2: 0.8±0.3 fmol/mg). Incubation of preconfluent cells for 24 hr with 20 pM of human recombinant IL-1a did not modify the density of either of the betaAR subtypes. Similar incubations of confluent cells increased the density of beta2AR from 0.8±0.3 to 4.2±0.9 fmol/mg, while the density of beta1AR and the antagonist affinities of both receptors remained unaltered. The IL-1a-induced increase in beta2AR density in confluent cells was antagonized in a concentration dependent manner by a recombinant protein antagonist of type I IL-1 receptors (IC50: 0.2 nM). The IL-1a-induced increase in beta2AR density was preceded by an increase in the steady state level of beta2AR mRNA, while levels of beta1AR mRNA remained unchanged. The rate of transcription of the beta2AR mRNA was increased by IL-1a, as evidenced by the results of nuclear run-off assays.These observations indicate that activation of type I IL-1 receptors in A549 cells leads to a cell density-dependent, selective upregulation of beta2AR, and that the mechanism of this effect involves increased transcription of the beta2AR gene.

Project description

Investigators

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T. Szentendrei	Visiting Fellow	LPPS, NIAAA
L. Takacs	Visiting Associate	LPPS, NIAAA
M. Virmani	Research Chemist	LPPS, NIAAA

Objectives:

The characterize the subtypes of IL-1 receptors and ß-adrenergic receptors involved in the upregulation of ß-adrenergic receptors by IL-1 in cultured human lung cells, and to analyze the molecular mechanisms involved in this regulation.

Methods Employed:

Methods of cell culturing and of quantifying ßAR have been published in our previous papers on this topic. To estimate the relative and absolute densities and affinities of ßAR subtypes, the binding of 125I-pindolol and its displacement by subtype selective antagonists (betaxolol for ß1AR and ICI-118,551 for ß2AR) were analyzed for best fit to one or more binding sites using the Ligand program of Munson and Rodbard.

Total cellular RNA was extracted and purified by the guanidine thiocyanate method, using the RNAzol kit. For measuring the amount of ß2AR mRNA, we constructed a cDNA probe specific for the mRNA for the ß2AR. The method used to prepare the probe was as described in our paper in Endocrinology (see reference of Lazar-Wesley et al. following project Z01 AA 00403-04 LPPS). The cDNA probe used to quantify ß1AR mRNA was prepared and kindly provided by Dr. C.C. Malbon. The above study in Endocrinology also contains the details of the DNA-excess solution hybridization assay used to quantify steady state levels of ß1AR mRNA and ß2AR mRNA in A549 cells. The rate of receptor gene transcription was quantified by nuclear run-off assays. Nuclei from A549 cells were isolated by sucrose density gradient centrifugation. Nuclei were incubated for 30 min at 30°C in reaction buffer containing 35S-UTP. The radiolabeled RNA formed was purified by phenol/chloroform extraction. An equal number of counts from each sample were added to slot blots to which 1 µg of phage DNA containing the human ß2AR cDNA insert had been UV cross-linked. After hybridization for 72 hr at 42°C, the filters were washed in 2 x SSC, 0.1% SDS at 42°C and then at 55°C, and were exposed in a Phosphorimager cassette. The screens were scanned and the bands were quantified by density integration.

Major Findings:

1. There are at least two distinct types of cellular receptors for IL-1. Recently, an endogenous protein has been cloned and characterized that

selectively antagonizes those actions of IL-1 that are mediated by type I IL-1 receptors (Eisenberg et al., Nature 343:341-6, 1990). Using this recombinant antagonist (kindly provided by Dr. Eisenberg) we found that it potently antagonized the upregulation of ßAR by IL-1α in A549 cells (IC50: 0.2 nM). This and our earlier finding that IL-1α and IL-1ß are equipotent for this effect indicates that the effect of IL-1 is mediated by a type I IL-receptor in A549 cells.

2. The results of computer-assisted curve-fitting experiments indicate that both ß1AR and ß2AR are expressed in A549 cells, and that the expression of the ß2AR subtype is cell density-dependent: the cells lose most of their ß2AR upon reaching confluency. The results further indicate that upregulation of ßAR by IL-1 is selective for the ß2AR subtype, and this upregulation only occurs in confluent cells. Thus, the effect of IL-1 on ß2AR appears to be 'de-repression' of the inhibition of ß2AR expression by cell to cell contact, rather than a true upregulation.

3. Upregulation of ß2AR by IL-1 is preceded by a significant increase in the steady state level of ß2AR mRNA with no change in the level of ß1AR mRNA. The increase in ß2AR mRNA is related to an increase in the rate of transcription of the ß2AR gene, as demonstrated by the results of nuclear run-off assays. These results indicate that the most likely mechanism of the upregulation of ß2AR by IL-1 is an increase in the transcription of the ß2AR gene.

4. We continued work on the identification of IL-1-like proteins in conditioned medium from EBV-transformed human lymphocytes (IM9 cells) (J. Biol. Chem. 263:15876, 1988). Conditioned medium (CM) was concentrated by ultrafiltration and partially purified by ion exchange chromatography. Monoclonal antibodies have been raised against the partially purified CM. The antibodies have been immobilized on protein A columns, and screened for their ability to retain the biologically active component of CM passed through such columns. One antibody was found to reproducively retain 50-80% of the biological activity of CM, assayed as ability to increase ßAR density in A549 cells. This antibody is currently being expanded in mouse ascites for use in large-scale immunoaffinity purification of its antigen from partially purified CM.

Significance to Biomedical Research and the Program of the Institute

Pulmonary ßAR are involved in the maintenance of normal respiratory function (bronchodilation, surfactant production) and impaired respiratory function in certain diseases (e.g. bronchial asthma) has been associated with ß-adrenergic hypofunction and loss of pulmonary ßAR. Since IL-1 is produced locally in the lung by resident macrophages, its interaction with ßAR may have physiological importance in the control of respiratory function. Furthermore, IL-1 has been found to interact with a number of hormone-receptor systems, including some receptors, such as the GABA-A receptor, that have been implicated in the actions of ethanol. It is therefore possible that IL-1 may have a role in certain biological actions of ethanol.

Proposed Course:

1. We demonstrated an interaction between IL-1 and ß-adrenergic receptors in a human lung tumor cell line. In preliminary studies we found a similar interaction in human diploid fetal lung cells (MRC-9 cells). These studies will be extended to verify whether or not the interaction observed is limited to malignantly transformed cells.

2. The A549 cell line is derived from alveolar type II cells, and it has been used as a model system for the study of the hormonal regulation of pulmonary surfactant production. We will set up assays to quantify the production of surfactant lipid as well as apoproteins. Such assays will be used to test whether a) IL-1 itself may modify surfactant production, and b) whether IL-1 induction of ß2AR in A549 cells potentiates the well known ß-adrenergic effects on surfactant secretion. Such effects may be relevant in the pathophysiology of respiratory distress syndrome.

3. We will continue our efforts to purify and identify the IL-1-like factors described under #4 above.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00402-04 LPPS

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Brainstem Neuromechanisms in Blood Pressure Regulation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	G. Kunos	Laboratory Chief	LPPS, NIAAA
Others:	N. Gantenberg	IRTA Fellow	LPPS, NIAAA
	K. Varga	Visiting Fellow	LPPS, NIAAA

COOPERATING UNITS *(if any)*

Laboratory of Cell Biology, NIMH

LAB/BRANCH

Laboratory of Physiologic and Pharmacologic Studies

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NIAAA, 12501 Washington Avenue, Rockville MD 20852

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1.7	1.7	

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have extended our earlier, preliminary findings on the role of brainstem GABA in the ethanol inhibition of baroreflex bradycardia in urethane-anesthetized rats. Ethanol, 1 g/kg i.v. or 25-200 nmol microinjected bilaterally into the medullary dorsal vagal complex (DVC), inhibited the reflex bradycardic response to iv. phenylephrine in control rats but not in rats pretreated with the GABA-depleting agent, 3-mercaptopropionate. Ethanol, 1 g/kg iv., did not influence the bradycardic response to electrical stimulation of the cervical vagus. Intra-DVC injection of muscimol caused a pressor response and inhibited baroreflex bradycardia. The pressor response was potentiated and a tachycardic response to muscimol emerged following intra-DVC injection of ethanol. Intra-DVC injections of baclofen also caused pressor and tachycardic effects and inhibited baroreflex bradycardia. Intra-DVC bicuculline selectively inhibited the effects of similarly administered muscimol, and attenuated the baroreflex inhibitory actions of ethanol. Intra-DVC 2-OH-saclofen selectively inhibited the effects of similarly injected baclofen and also attenuated the effect of ethanol on the baroreflex. Although 2-OH-saclofen did not influence the responses to muscimol, it inhibited the potentiation of the muscimol response by ethanol. It is concluded that ethanol inhibits baroreflex bradycardia by potentiating the similar actions of endogenous GABA in the DVC. Both GABA-A and GABA-B receptors appear to be involved in this effect.

Project Description:

Investigators:

G. Kunos	Chief	LPPS, NIAAA
N. Gantenberg	IRTA Fellow	LPPS, NIAAA
M. Palkovits		LCB, NIMH
K. Varga	Visiting Fellow	LPPS, NIAAA

Methods Employed:

Male rats were anesthetized by urethane. The femoral vein and artery were cannulated for drug injections and direct measurement of blood pressure and heart rate, respectively. For intra-NTS injections, the dorsal surface of the medulla oblongata was exposed by limited craniotomy, and a glass microcannula was stereotaxically inserted into the NTS according to published coordinates. Baroreflex bradycardia was elicited by graded iv. doses of phenylephrine. Peak increases in blood pressure were plotted against the corresponding peak increases in pulse period, and the slope of the regression was used as an indicator of baroreflex sensitivity. For depletion of readily releasable stores of GABA, rats were injected ip. with 100 mg/kg 3-mercaptopropionate 15 min prior to testing drug effects.

Major Findings:

1. There is evidence that both GABA-A and GABA-B receptors are present in the DVC, and that they may both contribute to the pressor actions of endogenous GABA in the DVC. In earlier experiments we found that depletion of endogenous GABA completely prevented the baroreflex inhibitory action of ethanol, but antagonism of GABA-A receptors in the DVC by bicuculline only partially inhibited it. This could suggest that ethanol may interact not only with GABA-A receptors, but also with bicuculline-resistant GABA-B receptors in producing inhibition of the baroreflex. We confirmed this possibility in the present experiments in urethane-anesthetized rats. The role of GABA-A receptors was indicated by the ability of ethanol to potentiate the pressor and tachycardic effects of muscimol, and by the ability of bicuculline to antagonize both the pressor effects of muscimol and the baroreflex inhibitory action of ethanol. The role of GABA-B receptors was indicated by the ability of 2-OH-saclofen, a GABA-B receptor antagonist, to attenuate the baroreflex inhibitory action of ethanol. However, ethanol only marginally influenced the effects of baclofen. This raised the possibility that blockade of GABA-B receptors may interfere with the interaction of ethanol with the GABA-A receptor. This was confirmed by the finding that 2-OH-saclofen, which did not affect the control response to muscimol, prevented the potentiation of the muscimol response by ethanol.

2. The presence of interleukin-1ß (IL-1ß) and IL-1 receptors in the brain and the documented role of IL-1 in some forms of cardiovascular shock prompted us to examine the cardiovascular effects of IL-1ß. In extending earlier preliminary experiments we found that bilateral microinjection of 0.1-2 pmol of IL-1ß into the DVC causes dose-dependent bradycardia without consistent changes in blood pressure. IL-1α is at least 20 times less potent and also

less efficacious than IL-1ß. The bradycardic effect is completely blocked by methyl-atropine, indicating a vagal mechanism. The bradycardia can be also attenuated by iv. naloxone, which suggests the involvement of endogenous opioids. Finally, intra-DVC microinjection of a human recombinant protein antagonist against the typeI IL-1 receptor does not significantly affect the response to IL-1ß even at a 50-fold molar excess. In separate experiments described in project Z01 AA 00401-04 LPPS the same antagonist potently inhibited the IL-1-induced increase in ß-adrenergic receptor density in cultured human lung cells. These findings suggest that the DVC contains an IL-1 receptor not sensitive to the recombinant antagonist (such as the typeII 68kD IL-1 receptor also found in other areas of the brain), and that activation of these receptors results in increased vagal outflow to the heart via a pathway that has an opioid-mediated link.

Significance to Biomedical Research and the Program of the Institute:

The hypertensive and tachycardic effects of ethanol are well documented in the literature. Our findings indicate, for the first time, that an important cardiovascular effect of ethanol can be linked to ethanol's interaction with a specific neurotransmitter system, GABA interacting with both GABA-A and GABA-B receptors, at a specific site in the brain. These findings have potential implications for the drug treatment of hypertension in alcoholic individuals.

Proposed Course:

In our experiments to date the baroreflex was elicited by pressor doses of phenylephrine. This approach only allows the study of the vagal limb of the reflex. In order to also examine the possible effect of ethanol on the efferent sympathetic pathways, the baroreflex will be elicited by electrical stimulation of the aortic nerve. We will also further examine the potential physiological role of GABA-B receptor in the DVC. The hypothalamic defense area will be electrically stimulated to activate the postulated descending GABA-ergic pathway to the DVC. Microinjection of GABA-A and GABA-B antagonists into the DVC will be done to dissect the relative contribution of the two receptors to the pressor and tachycardic effects of locally released endogenous GABA. Finally, we will follow up a recent report indicating that IL-1 can potentiate certain GABA-A receptor mediated events. We will examine whether very low doses of IL-1 microinjected into the DVC can potentiate the pressor effects of similarly administered muscimol. Positive results may warrant further studies into the potential involvement of IL-1 in the interactions of ethanol with GABA-A receptors.

Publications:

Florentino A, Varga K, Kunos G. Mechanism of the cardiovascular effects of GABSA-B receptor activation in the nucleus tractus solitarii of the rat, Brain Research 1990;535:264-70.

Kunos G, Mastrianni JA, Mosqueda-Garcia R, Varga K. Endorphinergic neurons in the brainstem: role in cardiovascular regulation. In: Kunos G, Ciriello J, eds. Central Neural Mechanisms in Cardiovascular Regulation. New York: Birkhauser, 1991;122-35.

Hollosi M, Vargha H, Medzihradszky K, Fasman GD, Kunos G, Graf L. Structural determinants of the binding of gliadin fragments to human peripheral blood lymphocytes, Neuropeptides 1990;17:11-116.

Varga K, Kunos G. Ethanol inhibition of baroreflex bradycardia: Role of brainstem GABA receptors, Brit J Pharmacol 1990;101:773-5.

Varga K, Zakhari S, Kunos G. Neural mechanisms in the cardiovascular effects of ethanol. In: Kunos G, Ciriello, J, eds. Central Neural Mechanisms in Cardiovascular Regulation. New York: Birkhauser, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00403-04 LPPS

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Inverse Regulation of Hepatic α1 and ß-adrenergic Receptors.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	G. Kunos	Laboratory Chief	LPPS, NIAAA
Others:	E. Ishac	Visiting Associate	LPPS, NIAAA
	E. Lazar-Wesley	Senior Staff Fellow	LPPS, NIAAA

COOPERATING UNITS *(if any)*

State University of New York, Stonybrook (C. Malbon)

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Laboratory of Physiologic and Pharmacologic Studies

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☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Steady state levels of the mRNAs for alpha1B, beta1 and beta2-adrenergic receptors (alpha1B-AR, beta1AR, beta2AR) were quantified by DNA excess solution hybridization assays in the heart, lungs and liver of rats. Tissues for RNA extraction were obtained from euthyroid and thyroidectomized rats and from thyroidectomized rats treated with a single dose of thyroxine. Thyroidectomy resulted in significant decreases in beta1AR and beta2AR mRNAs in heart and lung and alpha1B-AR mRNA in liver, whereas the levels of beta2AR mRNA in liver and alpha1B-AR mRNA in lung and heart were significantly increased. All these changes were reversed within 20 hours of a single s.c injection of 1 mg/kg thyroxine. These findings indicate for the first time that thyroid state regulates mRNA levels for adrenergic receptors, and that this regulation is tissue- and receptor-specific. The changes in adrenergic receptor mRNAs correlate with and probably underlie the well documented, thyroid-dependent changes in the cellular densities and physiological reactivities of adrenergic receptors. Steady state levels of alpha1B-AR and beta2AR mRNAs were also quantified in freshly isolated rat hepatocytes and in hepatocytes incubated in vitro in Krebs buffer for 2-24 hr. A significant decrease in alpha1B-AR mRNA and a parallel increase in beta2AR mRNA was detected as early as after 2 hr of incubation of cells. These changes precede and are probably related to the well documented time-dependent conversion from α1- to ß2-adrenergic glycogenolysis in isolated hepatocytes.

Project Description:

Investigators:

G. Kunos	Chief	LPPS, NIAAA
E. Ishac	Visiting Associate	LPPS, NIAAA
E. Lazar-Wesley	Senior Staff Fellow	LPPS, NIAAA
C. Malbon		SUNY, Stonybrook, N.Y.

Methods Employed:

Phosphorylase a activity was measured by the incorporation of 14C glucose-1-P into glycogen. Cyclic AMP levels were determined by RIA, IP3 levels were determined by a competitive protein-binding assay.

Preparation of cDNA probes and the DNA excess solution hybridization assays for the α1B, β1, and β2AR have been described in the reference by Lazar-Wesley et al.

The density and affinity of α1AR and β2AR were quantified in freeze-thawed, whole cells by the specific binding of 3H-prazosin and 125I-pindolol, respectively.

Major Findings:

1. There is substantial literature on the inverse regulation of α1 and β2AR in various tissues under different physiological and pathological conditions (see Biochem Pharmacol. 36:1185-91, 1987). Among the hormonal factors that have been shown to induce such changes are thyroid hormones and changes in thyroid state. Hypothyroidism in the rat was shown to reduce β- and enhance α1-adrenergic reactivity in the heart and some other tissues, while opposite changes were observed in the liver. In general, binding site densities change in parallel with the altered physiological reactivity. In the present experiments we demonstrate changes in the steady state levels of α1-, β1- and β2-AR mRNAs in hypothyroid rats that closely correspond to the earlier findings of altered receptor reactivities and cellular densities. Namely, hypothyroidism decreases the steady state level of α1AR mRNA in the liver and increases it in the heart and lung, while the level of β2AR mRNA is increased in liver and decreased in heart and lung. The mRNA for β1AR changes in the same way as that for β2AR, except that β1AR is undetectable in liver. The role of thyroid hormone in these changes is indicated by the ability of a single dose of thyroxine to reverse all these changes to or toward levels in controls. These findings are the first evidence that thyroid hormones are transcriptional regulators of adrenergic receptors, and that this regulation is tissue and receptor specific.

2. The most attractive model for studying the mechanisms involved in the inverse regulation of α1- and β-receptors is the primary culture of isolated rat liver cells, as the changes in this system develop in vitro over a very short period of time (hours compared to days or weeks in hypothyroidism). We used DNA excess solution hybridization assays to quantify time-dependent changes in the steady state levels of α1- and β2AR mRNAs in isolated rat liver

cells. Within 4 hours of primary culturing the levels of ß2AR mRNA more than doubled and the level of α1BAR mRNA decreased by 50%. Time course studies indicate that the increase in ß2AR mRNA increases very rapidly (peak at 1 hr) and precedes the more gradual decrease in α1BAR mRNA. In the same cells, the density of α1AR receptors decreased significantly after 4 hr of incubation, while the density of ß2AR increased only slightly and not significantly. However, when protein synthesis was blocked by cycloheximide, the density of ßAR decreased with time, suggesting that proteolytic degradation may mask the time-dependent increase in the synthesis of new ßAR. These findings suggest that the time-dependent conversion from α1- to ß-adrenergic glycogenolysis in isolated rat hepatocytes is related to inverse changes in the transcription of the genes for α1AAR and ß2AR. Experiments in progress are aimed to determine for both receptor whether the altered steady state levels of the mRNAs are related to altered rate of transcription, altered stability of the message, or both.

Significance to Biomedical Research and the Program of the Institute:

Inverse changes in α1- and ß-adrenergic receptors represent one form of 'cross-talk' between cAMP- and Ca2+-linked receptor systems. This unique form of receptor regulation has been documented in a variety of disease conditions, such as bronchial asthma, cystic fibrosis and essential hypertension. Understanding the molecular nature of this form of receptor regulation may thus contribute to our understanding the pathophysiology of certain diseases.

Proposed Course:

1. An important question to resolve is the nature of the primary signal that is activated by loss of cell-to-cell contact and then triggers the specific changes in the transcription of the adrenergic receptor genes. Preliminary experiments suggest that one such factor may be hepatocyte growth factor that acts on the c-met protooncogenes as its receptor. Further experiments will be done to substantiate this possibility. These studies will involve neutralization of endogenous HGF action by antibody, and measurement of the expression of the HGF and c-met genes during primary culturing of hepatocytes.

2. Earlier studies have indicated that activation of protein kinase C and generation of an arachidonate product during primary culturing of hepatocytes are causally involved in the shift from α1- to ß-adrenergic responses. In light of the current findings of early changes in the transcription of the genes for these two receptors, we will examine the possibility that PKC and arachidonate metabolites may be involved in the regulation of the transcription of α1BAR and ß2AR genes.

Publications:

Grojec SM, Ishac EJN, Kapocsi J, Kunos G. The effect of essential fatty acid deficiency on the adrenergic activation of glycogenolysis in rat hepatocytes, Arch Biochem Biophys 1990;283:34-9.

Lazar-Wesley E, Hadcock JR, Malbon CC, Kunos G, Ishac EJN. Tissue-specific regulation of α1B-, ß1-, and ß2-adrenergic receptor mRNAs by thyroid state in the rat, Endocrinology, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00486-01 LPPS

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Mechanism of Ethanol on GABA-Channels in Neurons

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	L. Reinlib	Senior Staff Fellow	LPPS, NIAAA
Others:	G. Kunos	Chief	LPPS, NIAAA

COOPERATING UNITS *(if any)*

None

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  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

GABA is the major inhibitory neurotransmitter in the CNS. It has been suggested that ethanol exerts its inhibitory effects on the CNS by potentiating the activity of the GABA-A receptor-gated chloride channel. However, electrophysiological and isotopic studies have provided conflicting evidence regarding the effects of ethanol on GABA-A activity. In an attempt to clarify this controversy and to gain further insight into the effects of ethanol on the function and regulation of the GABA-A channel, we have measured chloride flux in primary cultures of rat cerebellar neurons using the intracellular fluorescent probe, SPQ. Single cells were studied using a microscope - computer - photomultiplier system in order to characterize GABA-A responses within the population of viable cells. Chloride efflux from the cell down an imposed concentration gradient was observed over time. GABA dose dependently increased the rate of chloride efflux. The GABA response was sensitive to the GABA-A antagonist, bicuculline. Preliminary studies involving alcohol suggest that exposing the cells to GABA in the presence of physiologically relevant concentrations of ethanol further increase chloride efflux. Recent reports have suggested that cAMP-dependent phosphorylation inhibits GABA-A activity. Whether the mechanism of ethanol on the GABA channel is through cAMP-dependent pathways is currently under investigation.

Project Description:

Investigators:

L. Reinlib	Senior Staff Fellow	LPPS, NIAAA
G. Kunos	Chief	LPPS, NIAAA

Methods:

Neonatal rat cerebellar granule cells were isolated by trypsinization and maintained in culture. The cells were loaded with the fluorescent indicator, SPQ. Cells were mounted in a closed chamber and perfused with a solution containing (mM) NaCl 145, KCl 25, Na2HPO4 1, MgC12 1, CaC12 1, glucose 10, HEPES 10, pH 7.40. The SPQ-loaded cells were excited with 358 nm light and were observed on the stage of a Nikon Diaphot-TMD inverted microscope fitted with an adjustable aperture and photomultiplier. The system was assisted by an NEC 286 computer and software by Photon Technology International, Inc. In order to observe chloride efflux, the perfusing media was changed within 3 seconds to chloride free solution. GABA and drugs were added directly to the chloride free solution. The rate of chloride efflux in the presence of a particular agent was compared to a control perfusion in the same cell of chloride-free media without addition.

Major Findings:

1. Chloride efflux is mediated by GABA in the cultured cerebellar granule cell. Approximately 80% of the cells increased the rate of chloride efflux when exposed to GABA at concentrations between 1-100 x 10^{-6} M. The EC50 for GABA stimulation was 8.1 x 10^{-6} M. The selective GABA-A antagonist, bicuculline (10^{-4}M), completely blocked GABA-induced chloride flux at 7.5, 50 and 100 x 10^{-6} M GABA. The results provide support for the use of the cultured cells system as a model to investigate the function of the neuronal GABA channel.

2. Low concentrations of ethanol (<10 mM) increased the rate of GABA-induced chloride efflux by approximately 50% over control.

3. Preliminary experiments suggest that the GABA channel is regulated by cAMP-dependent mechanisms. The phosphodiesterase inhibitor, theophylline, was used to increase the level of cAMP in granule cells. Under these conditions, GABA-induced stimulation of chloride efflux was reversed. The results support the hypothesis of regulation of the GABA channel by a phosphorylation event.

Significance to Biomedical Research and the Program of the Institute:

GABA is the major inhibitory neurotransmitter in the central nervous system. Current work has demonstrated potent effects of ethanol on GABA receptor-linked chloride movements. There is also data which suggests that tolerance to ethanol may be a result of an adaptation of the GABA-A chloride channel. Our investigations define a technique which has not been applied to the neuronal system. These studies represent an important step to understanding the mechanism of tolerance and the sedative effects of ethanol.

Proposed Future Investigation:

Although regulation of the GABA channel through second messengers and phosphorylation has been reported, it is unknown whether ethanol may modify such regulation. Researchers have been unable to demonstrate an effect of ethanol on the phosphorylation of exogenously added substrates. Our further studies will determine whether ethanol influences the effect of theophylline on GABA-induced chloride movement. In addition, the role of protein kinase C on GABA-activated chloride efflux will be evaluated in the presence and absence of biologically relevant concentrations of ethanol. Further work will focus on endogenous substrates whose phosphorylation by cAMP and protein kinase C may be altered by ethanol. The substrates will be identified by use of [^{32}P] incorporation and autoradiography. These studies will extend previous work and help in the understanding of GABA channel function and the influence of ethanol.

Electrophysiological studies have indicated that ethanol potentiates the GABA-gated chloride current in some but not in other neurons. It has been hypothesized that such selectivity in the effects of ethanol is related to differences in the subunit composition of GABA-A receptors in various brain regions. Recent neurophysiological studies in the rat have indicated that the well known inhibition of the baroreceptor reflex by ethanol is related to a potentiation by ethanol of the actions of endogenous GABA at the brainstem nucleus of the solitary tract (NTS). It was also found that both GABA-A and GABA-B receptors are involved in this interaction (see project Z01 AA 00402-04 LPPS). Therefore, we will examine in in vitro preparations of this brainstem region whether ethanol can potentiate the GABA-gated chloride flux, as it does in cerebellar neurons. This preparation will also be used to test potential interactions between GABA-A and GABA-B receptors, suggested by the above neurophysiological studies.

Publications

Reinlib L, Akinshola E, Mezey E. Ethanol-induced increases in [Ca^{2+}]$_i$ and inositol(1,4,5)triphosphate in rat hepatocytes, Biochem Biophys Res Commun 1990;173:774-80.

Cohen ME, Reinlib L, Watson AJM, Corelick F, Rys-Sikora K, Tse M, Rood RP, Czernik AJ, Sharp GWG, Donowitz M. Rabbit ileal villus cell brush border Na^+/H^+ exchange is regulated by Ca^{2+}/calmodulin-dependent protein kinaseII, Proc Nat Acad Sci 1990;87:8990-4.

Mikkelsen RB, Reinlib L, Donowitz M, Zahniser D. Hyperthermia effects on cytosolic [Ca^{2+}]$_i$: Analysis at the single cell level by digitized imaging microscopy and cell survival, Cancer Res 1991;51:359-64.

Reinlib L, Homaidan F, Watson A, Bredt DS, Sharp GWG, Zahniser D, Donowitz M. Uncoupling of phospholipase C from receptor regulation of $[Ca^{2+}]_i$ in T_{84} colonic cells by prolonged exposure to phorbol dibutyrate, J Biol Chem, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00481-02 LPPS

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Regulation of IL-1 Production

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	L. Takacs	Visiting Associate	LPPS, NIAAA
Others:	L. Bristol	Staff Fellow	LPPS, NIAAA
	E. Romm	Chemist	LPPS, NIAAA

COOPERATING UNITS *(if any)*

Laboratory of Cell Biology, DCBD, NCI (E. Appella, S. Moore); Division of Cancer Treatment, NCI (S. Durum); Laboratory of Viral Carcinogenesis, DCE, NCI (N. Yuhki)

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

IL-1 is a major pro-inflammatory cytokine that induces HIV transcription in macrophages. The understanding of the regulation of IL-1 production would lead to possibilities to suppress its undesirable effects. Earlier we have purified a novel factor that induces IL-1 production in macrophages. Based on the N terminal sequence several unsuccessful attempts were made to clone this factor from cDNA libraries. The failure of these experiments is probably due to the degeneracy of the nucleotide sequence deduced from the protein sequence. HIV infection induces dementia in many patients. This dementia is related to the presence of HIV infected macrophages in the CNS. What causes the initial transcriptional activation of HIV in the brain, or the migration of infected cells, is now known, but it is possible that this is due to the presence of pro-inflammatory cytokines. Therefore we initiated studies to analyze the expression of pro-inflammatory cytokine genes (IL-1, TNFalpha) in the mouse brain. The results suggest that under basal conditions these genes are transcribed at low levels, detectable only by PCR analysis. However, i.v. exposure of the animal to known inducers of inflammatory cytokine production has markedly increased the levels of IL-1 and TNFalpha mRNAs strong stimulatory effect in all of the brain areas studied. These results suggest that pro-inflammatory cytokine production in the brain might be strongly affected by inflammatory processes elsewhere. Thus it is possible that careful anti-inflammatory treatment of AIDS patients would reduce the speed of dementia development.

Project Description:

Investigators:

L. Takacs	Visiting Associate	LPPS, NIAAA
E. Appella	Section Chief	LCB, DCBD, NCI
L. Bristol	Staff Fellow	LPPS, NIAAA
S. Durum	Senior Investigator	DCT, NCI
S. Moore	Senior Staff Fellow	LCB, DCBD, NCI
E. Romm	Chemist	LPPS, NIAAA
N. Yuhki	Visiting Fellow	LVC, DCE, NCI

Objectives:

IL-1 is believed to play a central role in the regulation of the inflammatory process and immune response. Previously by in situ hybridization analysis we found that IL-1 genes (alpha and beta) are transcribed in a small subset of tissue macrophages throughout the body in normal mice derived from a pathogen free environment. However, most of the macrophages did not express IL-1 genes. Theoretically, induction of IL-1 gene expression could be due to two major factors, exposure to bacterial products, or physiological regulators that induce IL-1 gene expression and IL-1 production. The objective of this project is to analyze these factors

A: Previously we have characterized a cytokine that is produced by mouse T-cells and induces IL-1 production in peritoneal macrophages. Based on another biological effect of this factor, the induction of spreading, the factor was purified to homogeneity and the sequence of a 20 amino acid N-terminal fragment was determined. During the past year we attempted to clone the cDNA of this factor, because a Gene Bank search showed no evident homology to other cytokines.

B: The role of LPS and exogenous IL-1 inducing factors were analyzed in several tissues in "in vivo" experiments.

By in situ hybridization we did not find IL-1 gene expression in the normal brain. However, by immunohistological analysis Saper et. al. described IL-1 beta protein in several areas believed to be involved in thermoregulation and sleep.To resolve this apparent enigma we used the PCR technique to amplify IL-1 genes from the brain from normal animals and from mice following intracerebral and i.v. LPS injection.

Methods Employed:

Library construction and screening: pA+ RNA was purified from stimulated mouse spleen cells and cDNA was synthethized from this by random priming. The double stranded cDNA was cloned into Lambda Zap vector according the instructions of the manufacturer (Stratagen). The unamplified library was plated and screened with standard methods with end labelled degenerate oligonucleotide probe (53 mer) that was synthethized based on the N-terminal sequence of the factor. Several libraries were generated form different cell and tissue types.

Northern analysis: RNA was isolated by gradient centrifugation over Cesium-trifluoroacetate from citrate buffered guanidium isothiocyanate lysates of cells. Total RNA was electrophoresed in formaldehyde gels were blotted to Nytrane membranes and hybridized to double stranded cDNA probes labelled by random priming.

Isolation of polyadenylated RNA : pA+ RNA was purified over oligo dT columns by standard techniques.

PCR amplification from pA+ RNA : Single stranded cDNA was transcribed from pA+ RNA by reverse transcription with oligo dT primer. About 1 kB fragments of both IL-1 alpha and beta were amplified from this cDNA in 50 cycles of reaction with the use of the recombinant Taq polimeraze. Products were run on non denaturing agarose gels, stained with ethidium bromide, photographed, alkali treated and blotted. Membranes were hybridized with double stranded cDNA probes labelled by random priming.

In situ hybridization: Sections were cut from frozen tissues in the cryostate. Immediately out from the cryostate sections were fixed in paraformaldehyde. Following a two step prehybridization sections were hybridized with ^{32}P labelled double stranded cDNA probes. Control sections were hybridized with the same probes, but the probe was not denatured before use.

Major Findings:

A: Several cDNA libraries were generated e.g. from LN427 cells and two libraries from stimulated spleen cells. These libraries were screened at least two times with a degenerate oligonucleotide mixture. No specifically hybridized clones were found.

B: IL-1 has CNS effects, in a recent report by Saper et. al. described IL-1 in neurons by immunohistology localized to areas involved in thermoregulation and sleep. We did not find IL-1 expressing cells in the normal brain by in-situ hybridization. To resolve this apparent enigma IL-1 expression was studied in the normal and LPS injected brains of mice by PCR analysis.

The normal brain did not contain IL-1 mRNA in the initial experiments, however by improving the quality of the cDNA used in the reactions and using more cycles in the PCR reaction we could detect IL-1 transcripts in each studied area of the normal mouse brain. Following the injection of 200 ng LPS to the n.caudatus IL-1 expression was detectable within 3 hours. Similar results were obtained following i.v. injection of LPS.

Significance to Biomedical Research and the Program of the Institute:

A: Since the physiological mechanism of IL-1 induction is not known the cDNA cloning and expression of a novel cytokine that regulates IL-1 gene expression would be a significant finding.

B: HIV infection induces dementia in many patients. This dementia is related to the presence of HIV infected macrophages in the CNS.

What causes the initial transcriptional activation of HIV in the brain, or the migration of infected cell, is not known, but it is possible that this is due to the presence of pro-inflammatory cytokines. The results suggest that IL-1 and TNF genes are transcribed at low levels, detectable only by PCR analysis. However, i.v exposure to known inducers of inflammatory cytokine production has strong stimulatory effect in all of the brain areas studied. These results suggest that pro-inflammatory cytokine production in the brain might be strongly affected by inflammatory processes elsewhere. Thus it is possible that careful anti inflammatory treatment of AIDS patients would reduce the speed of dementia development.

Proposed Course:

A: We plan to complete the cDNA cloning and expression of the novel cytokine.

B: To answer the question whether external stimuli could be responsible for the basal level of IL-1 transcription in the normal brain, animals from germ free environment as well as embryo brains will be tested for IL-1 transcripts.

Publiciations:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AA 00482-02 LPPS

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Regulation of the Early Steps of T Cell Development

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	L. Takacs	Visiting Associate	LPPS, NIAAA
Others:	L. Bristol	Staff Fellow	LPPS, NIAAA
	A. Kurtz	Visiting Fellow	LPPS, NIAAA
	E. Romm	Chemist	LPPS, NIAAA

COOPERATING UNITS *(if any)*
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LAB/BRANCH
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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.3	1.0	0.3

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The objective of these studies is to analyze the early steps of T cell development in order to search for possibilities to increase T cell production in the thymus. An increased production of thymocytes would be helpful in the treatment of AIDS or in other diseases that cause T cell loss. A monoclonal antibody was generated to immature T cell blasts. This antibody has a co-stimulatory effect on the proliferation of immature T cells and bone marrow cells. The antigen recognized by the antibody was purified to homogeneity, and its N-terminal sequence analysis showed that the antigen is the dipeptidyl peptidase DPPIV, a membrane associated proteolytic enzyme. The identification of DPPIV as a hematopoietic and T lineage activator opens new possibilities in the analysis of immature T cell proliferation. Switching T cell development towards the gamma/delta lineage would be beneficial in HIV infected young patients, since these cells lack CD4, the HIV receptor. In order to achieve this goal experiments were initiated that analyze the regulation of TcR gamma chain expression and rearrangement. Another possible way to modulate T cell development is to regulate the thymic colonization process. We are in the process of the characterization of a T cell precursor invasion model.

Project Description:

Investigators:

L. Takacs	Visiting Associate	LPPS, NIAAA
L. Bristol	Staff Fellow	LPPS, NIAAA
A. Kurtz	Visiting Fellow	LPPS, NIAAA
E. Romm	Chemist	LPPS, NIAAA
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Objectives:

T-cells develop from bone marrow precursors in the thymus. During development a large number of potentially useful T-cells are generated and subjected to positive and negative selection to select those cells that do not react to self antigens but will be able to participate in immune responses against foreign or altered self antigens (viruses, tumors, bacteria etc.). The expression of the T cell receptor (TcR) discriminates these important steps of development from earlier events that do not require TcR expression. The early steps of intrathymic T cell development include colonization of the thymus by bone marrow derived precursors, proliferation of precursors cells and the upregulation of TcR gene rearrangement and expression.

The analysis of the early steps of T cell development is important for the design of a successful treatment to increase the production of T cells in HIV infected patients or in patients who suffer from T cell loss due to other causes e.g.
alcoholism.

Three main approaches were taken.

A: We have studied the regulation of proliferation of intrathymic T cell precursors the CD4-/CD8- (DN) cells and bone marrow cells with the help of a monoclonal antibody and cytokines that have co-stimulatory activity in proliferation assays.

B: Based on previous findings with IL-2 and IL-1 we initiated experiments to analyze the regulation the TcR gamma chain expression in early T cells.

C: A novel model of intrathymic traffic or precursor cell invasivity is under investigation.

Methods Employed:

Isolation of rat T-cell precursors: The DN cells were isolated by density gradient centrifugation followed by two steps of indirect panning.

Tissue culture: Cells were cultured with standard techniques in Click's medium supplemented with 10% FCS and antibiotics.

Proliferation assays: These assays were performed in 96 well plates (U bottom). Cultures were pulsed with 10-20 uCi 3HTdR/well and harvested in a PhD harvester.

Bone marrow colony assays: These assays were performed in semisolid medium containing 0.8% methylcellulose. As a source of cytokines conditioned spleen cell supernatant was used in several concentrations.

T-cell hybrids: purified rat DN cells were fused to BW 5147.G.1.4 cells in PBS containing 50% PEG 1500 and 10% DMSO. Hybrids were selected in HAT medium and screened for 1.3 positivity by cytofluorographic analysis. 1.3 positive hybridomas were subcloned by limiting dilution.

Cytofluorographic analysis and cell sorting: Cells suspensions were stained in 2% BSA, 0.02% NaN3 containing medium with saturating amounts of monoclonal antibodies that were detected by a polyclonal, secondary antibody conjugated to FITC. Samples were analyzed in the cytofluorograph. In some cases positive and negative cell populations were sorted and analyzed independently.

Northern analysis: RNA was isolated by gradient centrifugation over Cesium-trifluoroacetate from citrate buffered guanidium isothiocyanate lysates of cells. Total RNA was electrophoresed in formaldehyde gels, blotted to Nytrane membranes and hybridized to double stranded cDNA probes labelled by random priming.

Southern analysis: DNA was isolated from the same gradient as for RNA, digested with restriction enzymes and blotted to Nytrane filters in 0.5M NaOH. Filters were processed as given above.

Surface Iodination immunoprecipitation, "off diagonal" SDS PAGE: Cells were surface iodinated by the lactoperoxidase method. I^{125} labelled cells were lysed in detergent. The detergent lysates were incubated with Sepharose coupled protein A that was preincubated with monoclonal or polyclonal antibodies. The precipitate was eluted in SDS/PAGE loading buffer and analyzed under reducing or non reducing conditions. Off diagonal gels were run in a tube under non reducing conditions first, followed by a second dimensional analysis under reducing conditions. This technique allows the characterization of complex heterodimers.

Phosphorylation experiments: Western blots were prepared with 1.3 immunoprecipitates as given above. These blots were developed for phosphotyrosine containing proteins with a specific antibody and iodinated secondary reagent.

Affinity purification of 1.3 antigen: The cell surface associated protein recognized by the 1.3 mab. was affinity purified from detergent lysates (Triton-X 100) of rat lamina propria and from lysates of a rat thymic tumor cell RNK 16, grown in-vivo as an ascites. The first step of purification was a lectin chromatography utilizing ConA-Sepharose. The second step was an antibody affinity chromatography with 1.3 mAb covalently coupled to Sepharose 4B. The purified protein was run on SDS-PAGE gels and stained with silver nitrate. The fractions that showed apparent homogeneity were subjected for amino acid sequencing or used as purified proteins in enzyme assays (see later).

Ca++ mobilization studies: Cells (10^7/ml) were loaded with 3 μM Indo-1 acetoxymethyl ester (Molecular Probes, Eugene, OR) in RPMI 1640 at 37°C for 60 min. Indo-1-loaded cells, washed and resuspended (10^6/ml) in RPMI containing 2% human AB serum and 20 Mm HEPES, were maintained at 37°C until tested for calcium influx in response to specific mAbs, lectin or ionomycin. Calcium flux was monitored using an EPICS 753 flow cytometer with an Argon laser in UV mode to measure changes in the ratio of calcium-bound Indo-I (405 nm) to free Indo-1 (485 nm) versus time.

Enzyme assays: Neutral aminopeptidase activity was measured with Leu-pNA chromogenic substrate, while Dipeptidyl-peptidase activity was measured with Gly-Pro-pNA. In some assays a boronic acid substituted transition state analog dipeptide, Pro-boro-Pro was used as an irreversible inhibitor of DPPIV activity.

N-terminal amino acid sequencing: Gas phase microsequencing based on Edman degradation chemistry was used. This procedure was done by Kazuhiro Skagutchi and Ettore Appella at NCI.

Immunohistology: Sections were cut in the cryostat and fixed in acetone. Staining was performed in PBS with monoclonal antibodies followed by VECTASTAIN components.

The T cell receptor gamma clone is derived from a total thymic cDNA library prepared in a plasmid (pAT153) in the laboratory of A. Neil Barclay MRC Cellular Immunology Unit, University of Oxford, Oxford. Sequencing was done with Sequenase kit according to the instructions of the manufacturer.

Invasivity assay: $2x10^5$ - $5x10^6$, ^{3}HTdR labelled hybridoma cells were incubated with subconfluent adherent cell layers in T25 flasks. The cell that did not invade the spaces under the adherent cells were removed by vigorous washing. The culture was than lysed with 0.5M NaOH and harvested with a harvester. Triplicate samples were tested in each experiment.

Major Findings:

A: Identification of the 1.3 antigen as DPPIV (CD26) or Thymocyte activating Molecule-2

We have continued the characterization of the 1.3 monoclonal antibody that was generated against in-vitro cultured DN blast cells. From detailed analysis of the antigen expression in various tissues it became evident that bone marrow cells and T cell lineage cells express the 1.3 antigen. Interestingly the level of expression was high on some bone marrow cells and on DN cells but it was lower on more mature T cell populations.

In proliferation assays the 1.3 mab. served as a potent co-stimulatory signal for proliferation in the presence of IL-2 and PMA. The co-stimulatory potential of 1.3 showed a strong correlation with the level of expression on T cells. The stimulatory effect did not require cross linking. Since bone marrow cells expressed the 1.3 antigen, we analyzed whether colony formation in bone marrow cultures is stimulated by 1.3. In these experiments 1.3 had stimulatory effect on macrophage and granulocyte colony formation.

Because of the important and unique functional properties of the 1.3 antibody we decided to identify the surface antigen recognized by this monoclonal antibody.

SDS-PAGE analysis of detergent solubilized surface iodinated cells that showed positive staining with 1.3 in the cytofluorograph displayed a 1.3 precipitable heterodimer of 130 and 110 kD under non reducing conditions. Following reduction only the lower band was detected in one dimensional gels. However, in two dimensional off diagonal gels it became evident, that the 130 kD band becomes 110 kD, interestingly, a reduction released 20 kD protein was not detectable.

Thymocyte activating molecule (THAM) in the mouse which has very similar biological and biochemical properties to the 1.3 antigen, was shown to exhibit Neutral aminopeptidase (AP-N) activity, and a mAb generated against THAM was slightly inhibitory for the AP-N enzyme from a thymus extract. To test whether a 1.3 reactive cell type expresses AP-N activity and to determine if 1.3 mAb produced a similar effect on the AP-N in the rat, thymocyte lysates were tested for AP-N activity in assays utilizing Leu-pNA as a substrate in the presence of 1.3 mAb. AP-N activity in rat thymus lysate was not inhibited by increasing concentrations of 1.3 mAb or an isotype-matched control.

These data indicate that the 1.3 mAb does not recognize an enzymatic subsite on AP-N, but do not rule out that the 1.3 determinant is another epitope on AP-N. As a first approach in determining if an association exists between 1.3 and AP-N, we screened 1.3 positive and negative cells for AP-N mRNA by Northern blot analysis. The 1.3 positivity of a panel of different cell types did not correlate with AP-N mRNA expression, except in the case of the kidney. AP-N message was absent even in samples from the most brightly 1.3 staining cells; freshly isolated DN cells and a rat DN hybridoma, RDNH 24. Other lymphoid cells including thymocytes, a rat NK cell line and splenic blasts were also negative. Naquet et al. described high levels of surface expression of THAM or AP-N on the mouse BW 5147 cell line, whereas the 1.3 negative BW 5147.G.1.4 clone used in these experiments did not contain AP-N message detectable by northern analysis.

The partial or complete segregation of chromosomes often occurs during the subcloning of hybridomas. We probed Southern blots of genomic DNA from 1.3 positive subclones of RDNH 24 with rat AP-N cDNA to establish whether the 1.3 phenotype was related to the presence of the AP-N gene. 10 and 7 Kb Sst-1 restriction fragments of the AP-N gene are absent in the DNA of RDNH 24.4 and 24.5 subclones. The same size restriction fragments were detected in rat kidney-derived DNA, while the mouse BW cells did not show detectable fragments with this enzyme. Similar results were obtained with Bam HI digested DNA. Based on these findings we designated the 1.3 antigen, THAM-2.

The 1.3 antigen showed biochemical similarity to a novel rat DN cell antigen that induced ionized Ca influx when added to DN cells. In several combinations the 1.3 antibody did not induce Ca mobilization.

To identify the 1.3 antigen it was purified to homogeneity from the RNK 16 tumor. N-terminal sequence analysis demonstrated the N terminal 9 amino acid of the purified 1.3 antigen is identical to the amino acid sequence 28-37 present in the rat dipeptidyl peptidase (DPPIV). In addition another sequence was obtained that might represent a contaminating protein. In order to establish that the 1.3 recognizes the rat DPPIV. The antigen was purified from the rat lamina propria. This material had DPPIV activity that could be inhibited with a specific irreversible inhibitor of DPPIV, Pro-Boro-Pro. The positivity of DPPIV cDNA transfected CHO cells with the 1.3 antibody further established that it recognizes the rat DPPIV.

In order to establish the mechanism of action of 1.3 mab mediated stimulation, first we analyzed whether the enzymatic activity of DPPIV is altered in the presence of 1.3. The 1.3 mab did not affect DPPIV activity, moreover it had no effect on Pro-boro-Pro inhibition. The inhibitory peptide did not compete with the 1.3 mab on the cell surface. These results suggest that the 1.3 does not alter DPPIV's activity in the tissue culture system where it has potent costimulatory activity. Since the 1.3 immunoprecipitates contain two bands, and at this moment we are not able to answer the important question whether this represent two different forms of the same enzyme or two different proteins, out of which only one is DPPIV, we looked for alternative explanations.
In these experiments we searched for DPPIV associated phosphoproteins.

B: The role of cytokines, the regulation of TcR gamma chain transcription

In previous experiments we found that IL-2 has strong inductive effect on the transcription of the TcR gamma chain gene. In these experiments we found that freshly isolated DN cells contain an abundant mRNA that is unusually short (1.0 KB) compared to the mature mRNA that is 1.5 KB. IL-2 had strong effect on both transcripts.

We hypothesize that the short transcript is from non rearranged gamma chain gene that does not contain V region. The induction of transcription and subsequent rearrangement by IL-2 by first inducing this transcript in cells that are not yet committed to the gamma/delta lineage would explain our in-vitro results. IL-2 added to DN cultures selectively induces the development of gamma/delta cells.

Since similar short transcripts were not sequenced we obtained a TcR gamma cDNA clone with the desired characteristics, from total thymic cDNA library. This clone contains a correctly spliced TcR gamma C region sequence, upstream of this sequence there is a J region but instead of the variable region 5' from it there is a sequence from the J-C intron and the recombination recognition sequences. We are in the process of sequencing the 5' part of this cDNA and to establish whether this region is present in the short, early T-cell derived gamma chain transcripts.

C: A novel model of T-cell precursor invasivity or intrathymic traffic.

Thymocyte precursors are derived from the bone marrow. The circulating precursor enters the thymus by crossing the blood-thymus barrier. This barrier consist of the endothelium of the blood vessel, a perivascular connective tissue compartment that includes a basal membrane, and layer of perivascular fibrocytes (pericytes), the next layer is the thymic epithelium. On the other side of the epithelial cells there is another layer of basal membrane. In order to cross these layers the precursor cell has to recognize the thymus but at the same time have to express invasive properties.

During the process of DN cell hybridoma production we observed that some of the hybrid lines were growing under the DN preparation derived stromal cells of the thymus. We were unable to maintain the growth of the stromal cell lines, but could clone the hybrids.

Several rat fibrocyte and other types of adherent cells were obtained from ATCC. With these cell lines we performed experiments to measure the invasive capacity of the DN hybrid clones. An assay system that employs radiolabelled hybridoma cells was worked out. In this assay we could demonstrate that the RDNH22.3 cells have about 3-5 fold higher invasive activity (15% vs. 3%) than the Bw partner cells or another hybrid the RDNH22.4. The invasion was dependent on the number of adherent cells. The process showed saturation around 3h following the addition of the labelled hybridoma cells. The ratio of cells that show invasive property did not increase further, during 18h of incubation.

We also found that a small population of freshly isolated DN cells is invasive.

Significance to Biomedical Research and the Program of the Institute:

This is the first demonstration of the involvement of DPPIV in haematopoietic and T cell development. The results suggest that all of the cells could be costimulated that express this antigen. Thus there is possible mechanism that can be selectively targeted to increase haematopoietic and early T cell proliferation to compensate for the loss of peripheral T cells in AIDS or in other diseases.
By understanding the mechanism of TcR gamma chain expression and rearrangement and by the analysis of the effect of cytokines on this process it is possible that T-cell development could be switched to the gamma/delta lineage. These cells do not express CD4 antigen thus are more resistant to HIV infection.

Regulation of precursor cell invasivity is of key importance in T-cell development. It is possible that insight into this process will open new avenues for the pharmacological manipulation of T cell production.

Proposed Course:

A: The next important question is whether the bone marrow derived T-cell precursor express DPPIV or not. We plan to answer this question with the help of adoptive transfer experiments. Also we plan to digest several cytokines with purified DPPIV because many of these contain putative substrate sites for DPPIV, suggesting that one possible way of DPPIV's involvement in the regulation of hematopoietic and T proliferation is cleavage of soluble factors thus either activating or inactivating them.

However, the effect of the antibody (1.3) cannot be explained this way, therefore we plan to investigate this question by the continuation of the experiments to identify DPPIV associated membrane proteins.

B: In the future we plan to analyze whether the cDNA clone contains genomic non rearranged sequences that are present in the short gamma chain transcripts of precursor T cells. In case of positive answer we plan to identify the promoter and regulatory elements and to analyze whether cytokines influence their function. It is likely that these transcripts have regulatory role in rearrangement and in the decision whether the precursor cell will develop into the gamma/delta or the alpha/beta lineage.

C: First we'll try to answer the question why only a small (10-15%) of the hybridoma cells show invasive property and characterize the invasive subpopulation of T-cell precursors. Next we plan to identify specific genes whose presence are responsible for the invasivity of the model cell lines.

Publication:

Gotlieb HW, Takacs L, Finch LR, Kopp W, Weissman AM, Durum SK. CD8 gamma/delta cells: presence in the adult rat thymus and generation in-vitro from CD4-/CD8- thymocytes in the presence of IL-2, Cytokine, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00479-08 LPPS

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Synaptic Mechanisms and Ethanol Actions

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	F.F. Weight	Section Chief	LPPS, NIAAA
Others:	C. Li	Visiting Fellow	LPPS, NIAAA
	D.M. Lovinger	Staff Fellow	LPPS, NIAAA
	R.W. Peoples	NRC Fellow	LPPS, NIAAA
	S.R. Visentin	Visiting Fellow	LPPS, NIAAA

COOPERATING UNITS *(if any)*

None

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.4	4.1	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The cellular and molecular mechanisms that underlie alcohol's behavioral effects have not been established. This project studied the effect of ethanol on synaptic mechanisms using the whole-cell patch-clamp technique. Ethanol inhibited ion current activated by the glutamate agonist NMDA in a concentration-dependent manner over the concentration range 5 to 50 mM, a range that produces intoxication. The inhibition was not voltage- or use-dependent, was not due to an alteration of the ion selectivity of the ionophore and did not appear to involve an interaction with a regulatory site on the channel. The inhibition was correlated with the hydrophobicity of different alcohols suggesting a novel interaction with hydrophobic region of the NMDA channel. The inhibition was also correlated with the intoxicating potency of different alcohols, suggesting that the inhibition may contribute to the cognitive and neural impairments associated with intoxication. Ethanol inhibited kainate- and quisqualate-activated current in a concentration-dependent manner over the concentration range 50 to 200 mM, a range that produces general anesthesia. GABA-A activated ion current was potentiated by ethanol in some neurons and not affected by ethanol in other neurons. The concentration that potentiated GABA-A activated current was 10 to 50 mM. GABA-A current was also potentiated by benzodiazepines, suggesting that the augmentation of this current by ethanol may contribute to the anxiolytic actions of ethanol. Intoxicating concentrations of ethanol were also found to potentiate the ion current mediated by activation of the 5-HT-3 type of serotonin receptor. Behavioral experiments suggest that this effect may be associated with the recognition of ethanol action. Ethanol also produced a concentration-dependent inhibition of the ion current associated with ATP-gated ion channels over the concentration range 6 to 250 mM. The observations suggest that neurotransmitter-gated ion channels may be molecular sites of ethanol action in the CNS. In addition, the behavioral effects of ethanol may result from actions of ethanol on these receptor-gated channels.

Project Description

Investigators:

F.F. Weight	Section Chief	LPPS, NIAAA
C. Li	Visiting Fellow	LPPS, NIAAA
D.M. Lovinger	Staff Fellow	LPPS, NIAAA
R.W. Peoples	NRC Fellow	LPPS, NIAAA
S.R. Visentin	Visiting Associate	LPPS, NIAAA

Objectives:

The objective of this project was to elucidate the cellular and molecular mechanisms of alcohol's actions in the nervous system that underlie the behavioral effects of alcohol.

Methods Employed:

The role of neurotransmitter-gated ion channels in the actions of alcohol was investigated by studying the effect of ethanol on ion currents activated by various neurotransmitter agonists in dissociated and cultured neurons. The techniques used were briefly as follows.

1. Dissociated Neurons

Neurons were dissociated from the nodose, superior cervical and dorsal root ganglia, and from hippocampal and striatal regions of the central nervous system of adult rats using mechanical and enzymatic treatment (Ikeda et al., J. Neurophysiol. 55:527, 1986; Freedman and Weight, PNAS 85:3618, 1988). Electrophysiological recordings were obtained from these neurons within a period of ten hours after dissociation.

2. Cultured Neurons

Hippocampal and cortical neurons were dissociated from 16- to 17-day mouse or rat embryos and grown in culture using the method of Forsythe and Westbrook (J. Physiol. 396:515, 1988). Electrophysiological experiments were performed 2 to 4 weeks after plating.

3. Electrophysiological Recording

In the majority of experiments, membrane ion currents were recorded using the whole-cell patch-clamp method (cf. Ikeda et al. 1986). Receptor agonists and various pharmacologic agents were administered from a large-bore micropipette (>40 um tip diameter) placed near the cell soma. In experiments recording single channel currents, cell-attached patch-clamp recording was used and pharmacologic agents were in the patch-pipette (cf. Freedman and Weight, 1988).

4. Data Acquisition

The output of the patch-clamp amplifier was digitized and stored on magnetic media for subsequent analysis using a Compaq 386/20e microcomputer. The microcomputer was also used to generate various patterns of voltage commands.

Major Findings:

1. Ethanol Inhibition of NMDA-Activated Ion Current

The ion current induced by the glutamate receptor agonist N-methyl-D-aspartate (NMDA) in voltage-clamped hippocampal neurons was inhibited by ethanol. Inhibition increased in a concentration-dependent manner over the range 5 to 50 mM, a range that also produces intoxication. The amplitude of the NMDA-activated current was reduced 61 percent by 50 mM ethanol; in contrast, this concentration of ethanol had a relatively small effect on the amplitude of current activated by the glutamate receptor agonists kainate and quisqualate. The potency for inhibition of the NMDA-activated current by several alcohols was linearly related to their intoxicating potency, suggesting that alcohol-induced inhibition of responses to NMDA receptor activation may contribute to the neural and cognitive impairments associated with intoxication.

The mechanism of this ethanol-induced inhibition of NMDA-activated ion current has also been investigated. We found that the inhibition of NMDA-activated current by ethanol (50 mM) was similar over a range of membrane potentials from -60 to +60 mV, and ethanol (50 mM) did not alter the reversal potential of the NMDA-activated current. In addition, the percent inhibition produced by ethanol did not differ with NMDA concentrations from 10-100 uM, with glycine concentrations from 10-1000 nM (the range over which glycine potentiates the NMDA-activated current), or in the absence of presence of Mg_{2+} (10, 100, 500 muM), Zn_{2+} (5, 20 muM) or ketamine (2, 10 muM). The data indicate that ethanol does not reduce NMDA-activated ion current by voltage-dependent block of the ion channel, altering the ion selectivity of the channel, or altering the affinity of binding sites for NMDA, glycine or substances that regulate channel function. Taken together with the preceding data, these observations suggest that ethanol may inhibit NMDA-activated ion current by a novel type of interaction with a hydrophobic site on the NMDA channel.

2. Ethanol Inhibition of Kainate- and Quisqualate-Activated Current

In concentrations greater than 50 mM, ethanol produced a concentration-dependent inhibition of kainate- and quisqualate-activated current. This inhibition of non-NMDA glutamate receptor activated current continued to increase at ethanol concentrations greater than 100 mM, so that at a concentration of 200 mM the inhibition was greater than 45%. The concentration range of ethanol that significantly inhibited kainate- and quisqualate- activated currents corresponds to the concentration range of ethanol that produces general anesthesia. This suggested that ethanol-induced inhibition of kainate- and quisqualate-activated current may contribute to the general anesthetic effects of ethanol. We therefore investigated the action of two general anesthetics on excitatory amino acid activated currents, pentobarbitol and tricholoroethanol, the active metabolite of chloral hydrate. Pentobarbital inhibited kainate and quisqualate currents with similar potency (IC_{50} ~ 50 uM), but produced a greater maximal inhibition of the quisqualate current (70% inhibition vs. 55% for kainate). Pentobarbital blocked both kainate and quisqualate currents in a use-dependent but rapidly reversible manner, and thus may bind to a site within, or near the opening of the ion channels. Pentobarbital had essentially no effect on the NMDA current. Trichloroethanol inhibited ion currents activated by kainate and quisqualate currents much more potently than ethanol. The IC_{50} values for this inhibition were 2.9, 7.8 and 9.7 mM for NMDA, quisqualate and kainate, respectively. These observations are consistent with the hypothesis that inhibition of kainate- and quisqualate- activated responses may contribute to the general anesthetic effects of ethanol.

3. Ethanol Effects on GABA-Activated Cl^{1-} Current

The effects of ethanol on membrane ion currents activated by N-methyl-D-aspartate (NMDA) and gamma-aminobutyric acid (GABA) were studied under voltage-clamp conditions in isolated sensory neurons within hours of being dissociated from adult rats. The amplitude of the ion current activated by NMDA was decreased in the presence of 2.5-100 mM ethanol (IC_{50}, 10 mM), a concentration range that produces intoxication. The amplitude of the GABA-activated Cl^- current, on the other hand, was not significantly affected by this concentration range of ethanol.

The effects of ethanol on GABA-activated Cl^- current were also studied in cultured mammalian hippocampal and cortical neurons. Patch-clamp recordings revealed that 10-50 mM ethanol potentiated the Cl^- current in a concentration-dependent manner in approximately two-thirds of the cells studied. GABA-activated Cl^- current was also potentiated by benzodiazepines, suggesting that ethanol augmentation of this current may contribute to the anxiolytic actions of ethanol.

4. Ethanol Potentiation of 5-HT_3 Receptor-Mediated Ion Current

The preceding evidence suggests that receptor-linked ion channels may be targets for the actions of ethanol. We therefore studied the effect of ethanol on the ion current produced by activation of 5-HT_3 receptors. Ion current activated by 5-HT or the selective 5-HT_3 receptor agonist 2-methyl-5-HT in cells of the NCB-20 neuroblastoma cell line was potentiated in a concentration dependent manner in the presence of 25-100 mM ethanol. The rate of decay of 5-HT-activated current increased in the presence of ethanol, and the potentiation of current by ethanol was greater at low 5-HT concentrations (1 uM) than at higher concentrations (2-10 uM). 5-HT_3 antagonists block the discriminative stimulus effects of EtOH in pigeons suggesting that ethanol potentiation of neuronal responses to activation of 5-HT_3 receptors may be associated with the recognition of ethanol action.

5. Ethanol Inhibition of ATP-activated Current

The effects of ethanol on membrane ion currents activated by extracellular adenosine 5'-triphosphate (ATP) were studied in isolated bullfrog dorsal root ganglion (DRG) neurons by means of the whole-cell patch-clamp technique. Using Cs+ to replace K+ in the patch-pipette solution, the amplitude of the ATP-activated inward current was decreased by ethanol in a concentration-dependent manner over the range 6 to 250 mM. In this concentration range ethanol did not appear to have any other detectable effect, and did not change the reversal potential of ATP-activated inward current. The average inhibition of 5 uM ATP-activated inward current by 100 mM EtOH was 31±5.3% (SEM: n=8). The concentration that produced 50% inhibition (IC$_{50}$) was 160 mM. When using the normal patch-pipette solution, biphasic membrane current, i.e., inward followed by outward current was induced by ATP. Both current components were reduced in amplitude by ethanol. The results indicate that ethanol can produce a concentration-dependent inhibition of ATP-activated currents.

Significance to Biomedical Research and the Program of the Institute:

The cellular and molecular basis of alcohol's actions in the nervous system have not been established. The observation that pharmacologically relevant concentrations of ethanol can selectively affect the function of certain types of neurotransmitter-gated ion channels suggests that neurotransmitter-gated ion channels may be molecular sites of ethanol action in the CNS, and that the action of ethanol at these sites may underlie the behavioral effects of ethanol.

Proposed Course:

The action of ethanol on the function of neurotransmitter-gated ion channels will be investigated more extensively to determine the molecular mechanisms involved in these actions and whether ethanol affects other receptor-activated ion channels. The effects of tolerance and dependence on these effects of ethanol will also be studied. In addition, the actions of other psychoactive substances such as anesthetics, opiates and benzodiazepines will be characterized and compared to ethanol.

Publications:

Aguayo LG. Ethanol potentiates the GABAA-activated CL- current in mouse hippocampal and cortical neurons, Europ J Pharmacol 1990;187:127-30.

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White G, Lovinger DM, Peoples RW, Weight FF. Inhibition of N-methyl-D-aspartate activated ion current by desmethylimipramine, Brain Res 1990;537:337-9.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AA 00480-08 LPPS

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Nerve Cell Excitability and Ethanol Actions

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	F.F. Weight	Section Chief	LPPS, NIAAA
Others:	L.G. Aguayo	Senior Staff Fellow	LPPS, NIAAA

COOPERATING UNITS *(if any)*
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LAB/BRANCH
Laboratory of Physiologic & Pharmacologic Studies

SECTION
Section of Electrophysiology

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.4	1.2	0.2

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The mechanisms by which alcohol affects the excitability of the nervous system are poorly understood. This project investigated the cellular and molecular mechanisms involved in the regulation of nerve cell excitability and the effects of ethanol on those mechanisms. Excitability mechanisms were studied in adult rat dorsal root ganglion neurons cultured in the absence of serum and exogenously added nerve growth factor (NGF) using the whole-cell patch-clamp technique. Current-clamp recording revealed the presence of action potentials in these neurons that were inhibited by tetrodotoxin (TTX). With voltage-clamp recording, both an initial inward and a later outward current were observed. The inward current was pharmacologically separated into a transient sodium current and a sustained calcium current. The outward current was found to be a slowly activating potassium current that was inhibited by tetraethylammonium (TEA). The inward sodium current had a maximal amplitude near -10 mV and was completely blocked by TTX. In previous studies on DRG neurons in vivo and cultured in the presence of serum and NGF, both TTX-sensitive and TTX-resistant action potentials have been observed. By contrast, in this study only TTX-sensitive action potentials and TTX-sensitive sodium currents were found in neurons cultured in the absence of serum and NGF. The observations suggest that serum and/or NGF may regulate the expression of voltage-gated ion channels. In addition, the effect of ethanol was studied on several voltage-activated ion currents in different types of mammalian neurons; it was found to have little or no effect on these currents in concentrations of 100 mM or less.

Project Description

Investigators:

F.F. Weight	Section Chief	LPPS, NIAAA
L.G. Aguayo	Senior Staff Fellow	LPPS, NIAAA
W.C. de Groat	Professor	Univ. of Pittsburgh

Objectives:

Although it is well known that the administration of ethanol can affect nervous system excitability, the cellular basis of such actions is poorly understood. The objective of this project was to characterize at the cellular and molecular level the mechanisms regulating nerve cell excitability and the effects of ethanol on those mechanisms.

Methods Employed:

Excitability mechanisms were investigated by studying voltage-activated membrane ion channels in dissociated and cultured mammalian neurons. The techniques used were briefly as follows.

1. Dissociated Neurons

Neurons were dissociated from the nodose, superior cervical and dorsal root ganglia, and from hippocampal and corpus striatal regions of the central nervous system of adult rats using mechanical and enzymatic treatment (Ikeda et al., J. Neurophysiol. 55: 527, 1986; Freedman and Weight, PNAS, 85: 3618, 1988). Electrophysiological recording was obtained from these neurons within a period of ten hours after dissociation.

2. Cultured Neurons

Hippocampal and cortical neurons were dissociated from 16- to 17-day mouse or rat embryos and grown in culture using the method of Forsythe and Westbrook (J. Physiol. 396: 515, 1988). Electrophysiological experiments were performed 2 to 4 weeks after plating.

3. Electrophysiological Recording

In the majority of experiments, membrane ion currents were recorded using the whole-cell patch-clamp method (cf. Ikeda et al., 1986). Receptor agonists and various pharmacologic agents were administered from a large-bore micropipette (>40 um tip diameter) placed near the cell soma. In experiments recording single channel currents, cell-attached patch-clamp recording was used and pharmacologic agents were in the patch-pipette (cf. Freedman and Weight, 1988).

4. Data Acquisition

The output of the patch-clamp amplifier was digitized and stored on magnetic media for subsequent analysis using a Compaq 386/20e microcomputer. The microcomputer was also used to generate various patterns of voltage commands.

Major Findings:

1. Voltage-activated Currents

Whole-cell patch-clamp experiments on adult mammalian neurons acutely dissociated from nodose, superior cervical and dorsal root ganglia revealed a variety of voltage-activated membrane ion currents which include tetrodotoxin (TTX)-sensitive and TTX-resistant sodium currents, a low-threshold transient calcium current (LVA or T-type), a high-threshold sustained calcium current (HVA or L-type), a transient voltage-activated potassium current (A current), a sustained voltage-activated potassium current (delayed rectifier) and a sustained calcium-activated potassium current (C current). The proportion of these currents varied in different neurons and not all currents were found in all neurons. Ethanol, in concentrations of 100 mM or less, had little or no effect on these voltage-activated membrane ion currents.

2. Identification of Isolated Neurons

This study was undertaken to determine the feasibility of using axonal tracing techniques in combination with cell isolation and patch-clamp methods to examine the electrical properties of identified populations of neurons from the adult rat. Fluorescent dyes (fast blue, FB, 5% w/v and fluorogold, FG, 4% w/v) were injected into the wall of the urinary bladder to label visceral afferent and efferent ganglion cells in lumbosacral dorsal root ganglia (DRG) and major pelvic ganglia (MPG), respectively. In other rats, dyes were injected into the sciatic nerve and gastrocnemius muscle to label somatic DRG neurons. After 8-15 days for transport of dyes, neurons were mechanically and enzymatically isolated and studied within hours or after 1-10 days in culture. Similar numbers of FB and FG labelled cells were detected in intact ganglia; however, mainly FB cells were observed following the isolation procedure. Isolated FB neurons could be identified within 10 to 20 seconds of exposure to UV light (340 to 380 nm). Following such short exposure to UV light, the gross electrophysiological properties of the FB neurons were indistinguishable from those of unlabelled neurons. Both groups of neurons exhibited resting membrane potentials of -45 to -60 mV and action potentials of 80 to 110 mV. However, in the FB neurons, constant exposure to UV light for periods of 1 to 5 minutes produced a gradual increase in action potential duration, followed by a decrease in action potential amplitude. No changes in spike firing properties were observed in unlabelled neurons during a 5 minute exposure to UV light. These observations indicate that FB may be a suitable tracer for studying the electrical properties of identified neurons.

3. Regulation of Neuronal Excitability

In order to investigate neuronal excitability and its regulation, we have dissociated neurons from adult rat dorsal root ganglia and maintained them in tissue culture without serum or added growth factors. When cultured in serum-free growth factor-free media, neurons (15-30 um) survived for over 3 weeks and extended long processes. Whole-cell recordings revealed that the cells had resting membrane potentials negative to -50 mV. Application of depolarizing outward current steps elicited overshooting action potentials (duration <2.5 ms) in most of the cells examined. The action potential was blocked by the application of TTX (1 uM) but not Cd++ (500 uM). Replacing intracellular K^+ with Cs^+ greatly prolonged the duration of the action potential. Voltage-clamp recordings demonstrated the presence of inward and outward currents. From a holding potential of -100 mV, depolarizing steps activated a fast inward Na^+ current that was sensitive to TTX. With 75 mM external Na^+, this current activated at potentials positive to -45 mV and reached a peak at -15 mV. In the presence of internal Cs^+ (120 mM) and the absence of external Na^+, an inward Ca^{++} current was observed. Study of isolated outward currents from holding potentials of -50 mV and -100 mV revealed only a sustained K^+ current. This current was inhibited by 10 mM TEA. The results suggest that serum and/or growth-factors may regulate the expression of voltage-gated ion channels.

The effects of NGF (2.5 ng/ml for 1-2 weeks) on adult rat DRG neurons maintained in cell culture in defined-media without background cells were also investigated. Whole-cell recordings revealed no significant difference in resting membrane potential and input resistance between cells cultured in the absence (NGF^-) and presence (NGF^+) of NGF. The sensitivity of the Na^+ spike to TTX (1 uM) was different in cells cultured in the absence or presence of NGF. Spikes were abolished by TTX in 100% of NGF^- cells (n=15), while in NGF^+ cells the spike was abolished in only 41% of the neurons (n=29) ($p<0.001$, chi-square, x^2). The threshold for spike generation was significantly lower in NGF^- cells than in NGF^+ cells, -25 ± 1.1 mV vs -18.9 ± 2.2 mV ($p<0.05$, ANOVA), respectively. Voltage clamp experiments revealed that the action potentials were predominantly carried by TTX-sensitive and TTX-resistant Na^+ currents in the absence and presence of NGF, respectively. Chemosensitivity of DRG neurons was also different in the absence and presence of NGF. For example, the percent of neurons in which capsaicin (500 uM) current was detected increased from 19% (n=16) in NGF^- cells to 55% (n=29) in NGF^+ cells ($p<0.05$, x^2). The results suggest that NGF has trophic effects on voltage-dependent and chemosensitive properties in adult mammalian neurons.

Significance to Biomedical Research and the Program of the Institute:

The behavioral effects of ethanol and the development of dependence and tolerance to ethanol are due to the actions of ethanol on the nervous system. The cellular basis of such actions, however, is poorly understood. Characterization of the cellular mechanisms that regulate nerve cell excitability and the actions of ethanol on those mechanisms holds the promise of increasing our understanding of the cellular basis of ethanol's actions in the nervous system.

Proposed Course:

The mechanisms regulating nerve cell excitability will be characterized more fully, and the actions of ethanol on those mechanisms will be investigated more extensively. In addition, the actions of other alcohols and CNS depressants such as general anesthetics, opiates, and benzodiazepines will be characterized and compared to ethanol.

Publications:

Jerrells TR, Marietta CA, Weight FF, Eckardt MJ. Effects of adrenalectomy on ethanol-associated immunosuppression, Int J Immunopharmacol, 1990;12:435-42.

Aguayo LG, Weight FF, White G. TTX-sensitive action potentials and excitability of adult rat sensory neurons cultured in serum- and exogenous nerve growth factor-free medium, Neurosci Letts, 1991;121:88-92.

Weight FF. Cellular and molecular physiology of alcohol actions in the nervous system, Intl Rev Neurobiol, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00404-04 LPPS

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Control of calcium- and phosphorylation-regulated signaling pathways

PRINCIPAL INVESTIGATOR *(List other professionel personnel below the Principal Investigator.) (Name, title, laboratory, end institute affiliation)*

PI:	R.L. Kincaid	Section Chief	LPPS, NIAAA
Others:	S.K. Brining	Staff Fellow	LPPS, NIAAA
	S.C. Dixon	IRTA Fellow	LPPS, NIAAA
	P.R. Giri	Visiting Associate	LPPS, NIAAA
	S. Higuchi	Visiting Fellow	LPPS, NIAAA
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COOPERATING UNITS *(if any)*

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.8	4.4	0.4

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Molecular analyses of the catalytic subunit of the calmodulin (CaM)-dependent protein phosphatase (PrP), calcineurin (CN), were extended with identification of a non-neural cDNA isoform in mouse testis encoded by a separate gene. Anti-peptide antibodies were prepared against the three mammalian genes and their alternatively-spliced isoforms; these recognize at least 10 charge variants on two-dimensional gel electrophoresis. Such complexity in the catalytic subunit of this phosphatase may reflect a need for substrate specific, Ca2+-dependent dephosphorylation. Homologues of CaM-PrP were cloned from fungi (*Neurospora*, *Aspergillus*) and from the cellular slime mold (*Dictyostelium*), indicating a role for Ca2+-regulated phosphoprotein metabolism in lower eukaryotes. Studies of their expression in these organisms suggested a relationship to morphogenic and developmental events. In *Neurospora*, mRNA and immunoreactive protein correlated with the extent of mycelial branching, whereas in *Dictyostelium*, increased mRNA accompanied differentiation into pre-stalk and pre-spore cells. In primary neuronal cultures examined by immunocytochemical methods, it also appeared that this enzyme may be localized to structures in developing neurites. A recombinant catalytic subunit from *Neurospora* was produced with a p-nitrophenol phosphatase activity comparable to that of brain enzyme, although it showed a much different phosphoprotein substrate specificity; chimeric constructs with "variable" regions taken from mammalian genes are being prepared to analyze these differences. Ultrastructural e.m. studies of phosphodiesterase (PDE) revealed high levels in post-synaptic regions of assymetric synapses, with little staining in other parts of the dendrite, consistent with a role for PDE in integration of afferent input. A 36 kDa neuron-specific CaM-associated protein (NCAP-36), having phosphorylated isoforms *in vivo*, was localized to the synaptoplasm of thalamic and midbrain structures and was not detected outside of brain. Peptide microsequencing of proteolytic digests of NCAP-36 showed no homologies to other classes of enzyme.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

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J. Franke	Professor	Columbia Univ.
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K. Kosik	Professor	Harvard University
C.A. Marietta	Research Physiologist	LPPS, NIAAA
A.R. Means	Professor	Baylor University
M. Moos	Research Chemist	FDA (LMP)
T. Muramatsu	Visiting Fellow	LPPS, NIAAA
J.W. Polli	PRAT Fellow	LPPS, NIAAA

Objectives:

Calcium and cyclic nucleotides are known to mediate many intracellular responses elicited by effector or hormone stimulation. These second messengers regulate phosphoprotein metabolism in such "signaling pathways", and their interactions are often linked via the calcium binding protein, calmodulin (CaM). The primary goals of this project are 1) to characterize the roles of several CaM-dependent enzymes using immunological and molecular biological approaches, 2) to determine the regulation of, and interactions between CaM-, phosphoprotein- and cyclic nucleotide-dependent pathways during activation of neural and lymphoid cells, and 3) to investigate the involvement of these signaling events in adaptive and pathologic cellular responses to alcohol and to infective agents such as AIDS.

Methods Employed:

Purified IgG from rabbit (used for PDE immunocytochemistry) was made using conventional Protein-A Sepharose chromatography. Affinity-purified antibodies to PDE, CN and NCAP-36 were prepared by chromatography of crude rabbit antisera on columns of antigen immobilized to CNBr-activated Sepharose (1-3 mg protein/ml gel). Antibodies against synthetic peptides (Cambridge Research) were prepared by chromatography of crude sera over affinity columns of peptide conjugated to CNBr-activated Sepharose 4B (0.3-0.5 mg peptide/ ml gel). Elution of antibodies was carried out with denaturing buffer (50 mM sodium acetate, pH 4.8, containing 6 M urea, 500 mM NaCl and 10 mM EDTA) into 0.1 bed volumes of 2 M Tris HCl, pH 9.0; this solution was dialyzed versus 50 mM Tris HCl, pH 7.5,/ 150 mM NaCl (TBS) containing 40% glycerol to remove urea.

Immunocytochemistry was carried out using 70μ sections of rat brain that had been perfused with 10% formaldehyde; for fetal tissue, brains were immersed in fixative. After incubation with primary antibodies, the complexes were detected with peroxidase-conjugated second antibodies and stained using diaminobenzidine and hydrogen peroxide. For electron microscopy, stained sections were cut into 1 mm^2 blocks, post-fixed with 1% osmium tetroxide and imbedded in Epon. Thin sections were counterstained with lead citrate and uranyl acetate and examined under e.m.

Studies of the tissue distribution of CaM-binding proteins (BPs) were carried out using Western blot procedures with the above antibodies and/or biotinylated CaM (Bio-CaM), prepared as previously described. After blotting at a constant voltage of 100V, the nitrocellulose membrane containing immobilized proteins was incubated with primary antibody (or Bio-CaM) for 1-2 hr (26° C) followed by alkaline phosphatase- conjugated second antibody (or streptavidin) for 30-60 min; blots were visualized with the chromogenic substrates, nitro blue tetrazolium (NBT) and brom-chloro-indolyl phosphate (BCIP).

Cloning of cDNAs for CN from phage libraries was done using plaque hybridization to random-primed labeled biotinylated restriction fragments as described previously; for cloning from *Neurospora*, *Dictyostelium* or mouse testis libraries, full length mouse brain cDNAs prepared using polymerase chain reaction (PCR) procedures were used as probes. For cloning from an *Aspergillus* library, a probe was prepared using the *Neurospora* cDNA. After isolation of presumptive clones, phage DNA was subcloned into pUC-18 for conventional and automated DNA sequencing using the dideoxy nucleotide termination procedure. Radioactive probes for Northern blot analysis were prepared by Klenow-based labeling of the anti-sense strand using PCR-generated segments of the coding region and specific primers. RNA isolation was done using extraction with guanidine isothiocyanate followed by precipitation with $LiCl_2$; fractions were electrophoresed in 0.8% agarose gels containing 1% formaldehyde and transferred to membranes for Northern blot analysis. Analysis of DNA for sequence homologies was carried out using Gen-Bank with relational data bases prepared by commercial vendors (DNAStar).

Construction of plasmids for the production of recombinant proteins was carried out as follows: A portion of the open reading frame, corresponding to the region to be expressed, was amplified from a "parent" template using PCR. After removal of excess primers, the fragment was cloned into a Sma I site in pUC 18 using T-4 DNA ligase under conditions favorable for blunt end ligation (i.e., excess ligase, high ATP). The ligation was transformed into competent cells and suitable recombinants were selected after restriction analysis of miniprep plasmid DNA. Recombinant bacteria were then grown in 800 ml cultures, chromatographed on CaM-Sepharose, and the eluates analyzed for CaM-binding, immunoreactivity and phosphatase activity.

P-nitrophenyl phosphatase activity of brain CN or recombinant protein was measured using 20 mM PNPP, 5 mM $MgCl_2$, 10 µM $MnCl_2$, 100 µM EGTA and 150 µM $CaCl_2$ in the presence or absence of 0.4 µM calmodulin and/or other additions as indicated. Activity toward phosphoprotein substrates was measured under similar conditions, except that phosphoproteins substrates (inhibitor-1, histone, casein) were added to the assays at final concentrations of 0.5-2 µM. These substrates were phosphorylated using the catalytic subunit of cAMP-dependent protein kinase and 100 µM 32-P labeled ATP followed by exhaustive dialysis. Purified CN, PDE and NCAP-36 were prepared from fresh and frozen bovine brain as previously described. Briefly, batchwise DEAE chromatography at pH 6.0 was employed to separate pH 5.45-dependent (peak I) and 0.1 M NaCl (peak II) eluates containing predominantly PDE/NCAP-36 and CN respectively. These were then purified by CaM-Sepharose and Affi-Gel 501 chromatography, with an intermediate chromatography step on Cibacron Blue-Sepharose for peak II, in the case of CN.

Cultures of *Neurospora crassa* were grown by inoculation of conidia (5 x 10^5/ ml) in Vogel's medium supplemented with 1.5% sucrose or with glutamate. Mycelia were harvested by rapid filtration on filter paper, washed with distilled water and frozen on dry ice. Cultures of *Dictyostelium discoideum*, strain AX-4, were grown in standard completely defined medium and were harvested by low speed centrifugation. After washing with 20 mM Tris.HCl, pH 7.8, cells were frozen in dry ice.

Major Findings:

1) Regulation of cyclic nucleotide phosphodiesterase in brain. In a continuation of a project on regulation of rat brain PDE with Dr. Ludwig, electron microscopic studies were carried out. Previous studies at the light microscopic level had shown PDE to be expressed, in an apparently homogenous fashion, in the dendrites of specific output neurons. Also, in isolated subcellular fractions, PDE was not associated with synaptic membranes or junctional complexes. However, intra-dendritic compartmentalization of this soluble protein was observed at the e.m. level, with immunoreactivity localized to the post-synaptic side of assymetric synapses, rather than being distributed evenly in the cytoplasm. Immune reaction products were observed only rarely in regions of the dendritic shaft that were not innervated; this may represent PDE *en route* to a final destination or reflect the position of a developing synapse. The distribution of the regulatory subunit of type II cAMP-dependent protein kinase was compared in companion sections; this also showed a post-synaptic distribution although the number of neurons labeled was at least 5-10 times greater. These observations are consistent with the hypothesis that cyclic nucleotides play an important, if not predominant, role in post-synaptic function and that, in specific neurons, high concentrations of PDE are needed to regulate the levels of cyclic nucleotide in a discrete compartment near the junctional membrane. This latter relation may suggest that in some highly innervated central neurons, cyclic nucleotides have direct effects on membrane conductance, in addition to cytosolic actions mediated via protein kinases.

2) Analysis of protein phosphorylation in lymphocyte subpopulations. Previous studies had established that the predominant cytosolic CaM-regulated protein in murine lymphocytes was the protein phosphatase, CN. In lymphocytes, Ca^{2+}-dependent cascades are intimately associated with many recognition events, including the release of granules from cytotoxic T-cells by anti-T cell receptor activation. It was also well-known that protein phosphorylation in general plays a decisive role in many other lymphocyte signaling events (mitogenesis, lymphokine activation). In order to develop a comprehensive picture of the interplay between Ca2+-dependent and other signaling events *in*

vivo, Dr. Giri has initiated long-term studies that analyze the patterns of ^{32}-P phosphoproteins from different populations of lymphocytes and using various regimens of cell activation. By systematically varying incubation conditions with inhibitors of protein phosphatases, as well as with inhibitors and activators of the major classes of protein kinases, discrete phosphopeptides have been identified as substrates for the two classes of enzymes. For example, in both T- and B-cells, the phosphorylation of a major phosphoprotein band at 30 kDa was increased by incubation with phorbol ester, an activator of protein kinase C; this could be antagonized by 1 μM okadaic acid, an inhibitor of PrP-1, suggesting that the protein was regulated by these two enzymes. Using such a strategy, it should be possible to develop a "fingerprint" of a signaling pathway that outlines primary targets within the cell for different effectors and their down-regulation by phosphoprotein phosphatases. This method may permit the analysis of "signaling competency" in sera from patients that are immunocompromised, for example, in alcoholism or other disease states such as AIDS.

3) Molecular characterization of the catalytic subunit of CN.

In a continuation of previous molecular studies on the genetic diversity of CaM-PrP, additional cDNA and genomic clones have been characterized from mammalian (mouse, human) and lower eukaryotic sources. Previous studies from our section had compared the two major mammalian genes found in brain. Based on the apparent prevalence of these forms in brain, we assigned the nomenclature of gene 1 to the major form that we first isolated and gene 2 to the proline-containing form, originally described by Dr. C. Klee (NCI). These genes mapped to different human chromosomes (4 and 10, respectively) using Southern blot analysis of hamster human hybrid cell lines and a gene 2 probe hybridized only to mammalian DNA, whereas the homologous probe for gene 1 bound fragments from mammalian, avian and reptilian DNA. These results suggested either that gene 2 arose after the avian/reptilian branch point in evolution or that its sequence diverged much more rapidly than for gene 1.

Recently, Dr. Muramatsu in our group has cloned a new cDNA form from mouse testis that shows approximately 80% nucleotide sequence identity to both gene 1 and gene 2, indicating that it is derived from a separate gene (gene 3). In terms of its amino acid sequence, it appears slightly more related to gene 1 than gene 2 (86 vs. 83 % identity) and shows differences in the amino and carboxyl domains previously identified as variable regions in other CaM-PrP forms. Based on its deduced structure, it is approximately the same size (59 kDa) as the other gene products, but its predicted isoelectric point is much more basic (7.0) than those estimated for genes 1 and 2 (5.6-5.7); the significance of this rather large difference in net charge to questions of compartmentalization is not clear. The 2.8 kb mRNA for gene 3 was expressed in testis, while appreciable amounts were not seen in other tissues; thus, this appears to be the first non-neural form of CaM-PrP described at a molecular level. In work published 3 years ago, a form of this phosphatase was reported that appeared to be intimately associated with sperm flagella, suggesting a role in regulation of sperm motility. Studies using *in situ* hybridization are underway to determine the cellular localization within the testis and future studies with transgenic animals are anticipated, with the objective of assessing the role of this enzyme in reproductive function.

The existence of multiple mammalian catalytic subunits raises the issue of their role. To assess the possibility that these are localized to different cell types or to unique subcellular compartments, we have prepared anti-peptide antibodies against the three major classes of genes and against their isoforms that arise from alternative splicing. These reagents demonstrated 10-12 discrete immunoreactive species when purified brain CN preparations were resolved by high resolution two-dimensional electrophoresis; the isoelectric points of these ranged from 5.2-6.0. These forms were distributed differentially in different brain regions and several of these appeared to be associated with purified membrane fractions. A comparison of immunoreactive peptides with those observed on a silver-stained gel indicated that the major form (>70%) was the type 1 gene; antibodies that discriminate between the alternatively-spliced forms showed that those containing the highly acidic region of 10 amino acids were the major species. Antibodies against isoforms lacking this region recognized a minor series of four peptides having more basic pIs. Light- and electron-microscopic immunocytochemical studies have been initiated by Dr. Brining in our laboratory to address the potential differences in localization of these different isoforms. Also, a method has been developed to immunopurify these forms using the specific antibodies; this will allow us to clarify whether or not the catalytic properties of these CaM-regulated phosphatases vary significantly.

In collaborative studies with the laboratories of Dr. Kessin and Dr. Means, we have extended the work of Dr. Higuchi in our section on the characterization of cDNAs from lower eukaryotes. Previously, the catalytic subunit was cloned from the filamentous fungus, *N. crassa* and the structural conservation of the protein (≈ 70% identity to the mammalian forms) and the intron positions of its gene were detailed. This analysis predicted two things: 1) that there were several "variable regions" in the carboxyl terminus that might contribute elements of substrate specificity and 2) that there was a step-wise evolution of gene structure that conserved the positions of intron-exon boundaries. To address the first of these, an comparative analysis was carried out with a recombinant fungal catalytic subunit using phosphoproteins that were either general substrates (histone, casein) or highly specific ones for cAMP-dependent protein kinase (Inhibitor 1). These studies indicated that although the activity toward the generic substrates was not greatly different than that seen with brain CN, that toward Inhibitor-1, an important mammalian phosphatase substrate was negligible using the fungal enzyme. This supported the notion that the variable regions may contribute elements of substrate recognition, although it did not prove it. To provide more definitive information on this point, we are constructing chimeric recombinant subunits that contain the active fungal catalytic "core" fused to variable regions taken from the various mouse genes. The second area related to the evolution of gene structure was studied by cloning the corresponding genes from a slightly higher ascomycetes, *Aspergillus nidulans*, and from the slime mold, *Dictyostelium discoideum*. This work, which was carried out with Drs. Means and Kessin, respectively, showed that the fungal gene indeed contained a fourth intron in a position corresponding to mouse gene 1. However, the positions of two introns in the slime mold were not related; this suggests that the evolution of introns diverged in the earliest lines of eukaryotes but was maintained at least from fungi through mammals.

Another goal, separate from the comparative structural analysis of lower eukaryotic homologues of CaM-PrP, was the use of microorganisms as model systems to understand control of gene expression. We had previously demonstrated that the mRNA for CaM-dependent protein phosphatase in *Neurospora* increased markedly during the initial phases of mycelial growth. This finding was extended by analyzing the amount of immunoreactive protein using an antibody raised against the recombinant fungal catalytic subunit. The protein expression followed that of mRNA by about 8-10 hrs, peaking during a period of maximum mycelial branching, but then declined precipitously in spite of continued mRNA expression. These data indicate a tight control over the translation and degradation of the protein in relation to developmental events. In *Dictyostelium*, the amounts of mRNA for the phosphatase catalytic subunit were low at the beginning of morphogenesis, but they increased dramatically (6-8-fold) during the period of cellular differentiation into fruiting-body structures; we determined that this was not due to direct effects of cAMP on transcriptional activity, since CaM-PrP mRNA from suspension cultures incubated with cAMP were not elevated. Rather, the effect appears to relate to the specialization of discrete cell types. In cells physically isolated into pre-stalk and pre-spore types during the pre-culmination phase, there was dramatically more mRNA expressed in pre-stalk cells, again suggesting that a morphologic events was playing a role in controlling gene expression. Future studies using "knock-out" mutants in slime molds will be used to assess the importance of this enzyme in the morphologic and developmental behavior of this organism.

4) Regulation of phosphorylation-dependent signal pathways in model systems.

The CaM-dependent protein phosphatase, CN, is found in high concentrations in the striatum, a region receiving extensive dopaminergic innervation. It seems plausible that, as with the phosphodiesterase, expression of this enzyme *in vivo* may be regulated by the amount and/or type of neural innervation. In order to assess this possible regulation in whole animals, rats were treated by three separate paradigms- hemitransection of nigro-striatal pathways, neurotoxin destruction of afferent input and pharmacologic blockade of specific dopaminergic receptors. None of these treatments caused a significant change in the regional content of the protein phosphatase, although the possibility that small populations of cells were affected could not be ruled out. This finding, which was made in collaboration with Drs. Polli and Billingsley, suggests that dopaminergic innervation of the striatum and activation of D1 receptors does not play a major role in regulation of gene expression of CN.

Recent data from Dr. Giri in our group indicated that a phosphorylated form of CN was detectable in primary cerebellar granule cell cultures that have been incubated with ^{32}-P labeled inorganic phosphate. In order to pursue these initial findings and to address question of pharmacologic regulation of these glutamate-sensitive cells, Dr. Polli in our section has initiated studies to analyze the regulation of cAMP-dependent signaling events in these cells.

Workers in the laboratory of Dr. P. Greengard have implicated the phosphorylation and dephosphorylation of DARPP-32, a phosphoprotein inhibitor of type 1 PrP, in NMDA-dependent signaling events. Since an apparently spliced isoform of this protein has recently been cloned and characterized in our group by S. Dixon, and since the dephosphorylation of this protein is thought to be carried out by CN, we will examining the contributions of these proteins in the cellular responses to NMDA agonists and antagonists using a combination of immunochemical and molecular methods.

In a collaborative study with Dr. K. Kosik, the immunocytochemical localization of CN was determined in primary neuronal cultures in which axonal and neurite outgrowth can be distinguished. By examining the correspondence between the immunofluorescence of anti-CN and that of other structural proteins in the cells, this phosphatase appeared to be found in distal areas of extending neurites. Furthermore, the position of CN within the cell was altered by agents that disrupt cytoskeletal components, arguing for an association with such elements. In view of previous studies, from our group and others, on the apparent association of this enzyme with subcellular compartments, the findings from Dr. Kosik's laboratory imply that CaM-PrP is actively involved in the dephosphorylation of specific components that may regulate formation of developing neurites. It may be that these observations are related to some related role of this enzyme needed for the outgrowth of mycelial strands in fungi and the elaboration of pre-stalk cells in the development of slime molds. We have suggested before that mycelial outgrowth in fungi may provide a model system analogous to growth of neurons; we are presently attempting to determine the relationship between CaM-PrP immunoreactivity and that of structural components such as tubulin and acting during formation of mycelia in *Neurospora*.

5) Characterization of a NCAP-36, a novel brain-specific CaM binding protein.

Previously, we described a CaM-associated protein of subunit M_r ≈36,000 that we had purified approximately 3000-fold from bovine brain. We prepared an affinity-purified antibody against this protein and determined that it was present in a minor population of neurons in brain, specifically in thalamic and midbrain nuclei. Cross-reacting proteins were not observed in other tissues, even after enrichment by CaM-Sepharose chromatography, suggesting that this peptide is expressed uniquely in brain. Upon isolectric focusing of the purified protein, two major immunoreactive spots (50 and 40%) were observed with pIs of 6.2 and 6.0, respectively; a minor spot (10%) was seen with a pI of 5.85. Upon silver staining of these preparations, two spots were noted that had slightly lower mobility larger and were more acidic than the forms at pI 6.0 and 5.85. In studies carried out by Dr. Polli in our group, these spots were identified as phosphorylated forms of NCAP-36, since they were eliminated by alkaline phosphatase treatment and could be replicated by *in vitro* phosphorylation with cAMP-dependent protein kinase. Of the kinases tried, cA kinase and protein kinase C appeared to phosphorylate NCAP-36, reaching a maximum of 0.3-0.4 mol phosphate/mol substrate. This, along with the relative electrophoretic shifts seen, suggests that the isoforms capable of phosphorylation *in vivo* can incorporate phosphate at a single site. When the phosphorylation was carried out under conditions of excess CaM plus Ca^{2+}, the incorporation of phosphate was negligible, suggesting that CaM blocked this site either directly or by virtue of a conformational change. These data imply that phosphorylation of some isoforms of this protein may be influenced by (and may influence) interaction with CaM under physiologic conditions.

In order to identify the function of this protein, numerous enzyme assays were carried out. To date, NCAP-36 does not display PDE, phosphatase, GTPase, kinase activity. The protein was digested to completing with *S. aureus* V-8 protease and its peptides sequenced in collaboration with Dr. M. Moos. About 60% of the residues have been obtained; however, searches of all protein and nucleic acid databases have not yet identified regions of substantial similarity with other known proteins. Thus, the function of this protein is still unknown. We are currently carrying out the cloning of the protein using both immunoscreening and hybridization-based methods with degenerate oligonuleotides. By obtaining molecular clones and expressing these in model cultures, we hope to determine the role of this protein in cell function.

Significance to Biomedical Research and the Program of the Institute:

The biochemical responses to hormones, growth factors and other effectors are mediated by a variety of signaling pathways, some of which interact to influence each other. Several elements that play a role in signal transduction include Ca^{2+}/CaM- dependent enzyme activation, cyclic nucleotide "second messengers" systems and modulation of phosphoprotein metabolism. Investigation of the properties, distribution and regulation of these provides important information regarding normal and pathologic cellular activity and may lead to the design of rational pharmacologic therapies. Since chronic perturbation of the cellular environment resulting from drug or alcohol exposure may well lead to changes in signalling pathways, these studies may reveal fundamental features of pharmacologic dependence, tolerance and withdrawal.

Proposed Course:

To investigate the molecular biology of enzymes involved in Ca$_2^+$-dependent signal transduction (PDE, CN, NCAP-36) and to characterize their regulation during cellular activation and differentiation. To utilize specific immunological probes prepared against important regulatory sites, in order to understand the control of neural and immunomodulatory responses as well as modulation of intermediary metabolism. To examine signalling events under pathologic conditions (e.g. using murine models for alcoholism, inbred neuropathologies and immune disorders such as AIDS).

Publications:

Calalb MB, Kincaid RL, Soderling TR. Phosphorylation of calcineurin: effect on calmodulin binding, Biochem Biophys Res Commun 1990:172;551-6.

Kincaid RL. Signaling mechanisms in microorganisms: common themes in the evolution of signal transduction pathways, Adv Cyclic Nucleotide Prot Phosphoryl Res 1991:23;165-84.

Kincaid RL. Structural isoforms of the catalytic subunit of calmodulin-dependent phosphoprotein phosphatase ("calcineurin"): Deriving specificity by linking conserved and variable regions, Adv Protein Phosphatases 1991:6;73-98.

Ludvig N, Burmeister V, Jobe PC, Kincaid, RL. Electron microscopic immunocytochemical evidence that the calmodulin-dependent cyclic nucleotide phosphodiesterase is localized predominantly at postsynaptic sites in the rat brain, Neuroscience, in press.

Higuchi S, Tamura J, Giri PR, Polli JW, Kincaid RL. Calmodulin-dependent protein phosphatase from *Neurospora crassa*: Molecular cloning and expression of a recombinant catalytic subunit, J Biol Chem, in press.

Polli JW, Kincaid RL, Billingsley ML. Expression of calmodulin-dependent enzymes in developing rat striatum is not affected by pertubation of dopaminergic systems, Synapse, in press.

Polli JW, Billingsley ML, Kincaid, RL. Expression of the calmodulin-dependent phosphatase, calcineurin, in rat brain: identification of unique regional, developmental and subcellular patterns of expression using immunoblotting, immunocytochemistry and *in situ* hybridization, Develop Brain Res, in press.

Kincaid RL, Balaban CD, Billingsley ML. Regional and developmental expression of calmodulin-dependent cyclic nucleotide phosphodiesterase in rat brain, Adv Cyclic Nucleotide Prot Phosphoryl, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00405-04 LPPS

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Detection and Regulation of Specific Cellular Phosphoproteins

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	T.M. Martensen	Research Chemist	LPPS, NIAAA
Others:	R.L. Kincaid	Section Chief	USP, NIAAA

COOPERATING UNITS *(if any)*

Clinical Neuroscience Branch, NIMH (B. Martin)

LAB/BRANCH

Laboratory of Physiologic & Pharmacologic Studies

SECTION

Section of Immunology

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Ave., Rockville, MD 20852

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Phosphorylation of a Calmodulin (CaM)-dependent phosphoprotein phosphatase, calcineurin, (CN) by a CaM-dependent protein kinase modifies a specific serine residue. Dephosphorylation occurs by a mechanism in which the catalytic site of a modified polypeptide hydrolases its own phosphoserine bond. This autodephosphorylation can be regulated by effectors of CN enzymatic activity. Activators of the reaction were cations of Mn, Ca, and Ni. Ca/CaM, which stimulates intermolecular phosphatase activity, inhibits autodephosphorylation presumably by binding to a primary sequence of CN juxtaposed with the phosphoserine. Certain inhibitors of CN activated autodephosphorylation such as phenothiazine drugs (trifluoperazine, and chlorpromazine) and metal ion chelators (EGTA, and EDTA). In contrast to the finding of others found that phosphorylation inhibited the dephosphorylation of certain substrates, the phsophorylated enzyme dephosphorylated Inhibitor-1 at a more rapid rate than the dephosphorylated CN. A soluble enzymatic activity in brain exists that rapidly dephosphorylates phosphoCN. This activity is Ca/CaM independent and inhibited by nM concentrations of okadaic acid which is a characteristic of phosphoprotein phosphatase 2A. The ability to dephosphorylate phosphoCN is, however, dependent on denaturation of phosphoCN and maintenance of a low level of sodium dodecyl sulfate in the reactions. These features suggest the phosphoserine residue exists in a constrained structure which requires conformation change for facile dephosphorylation to occur. Antibodies obtained from rabbits immunized with synthetic peptides composed of conserved sequences found in CN and other phosphoprotein phosphatases bound radiolabeled CN. Antibodies affinity purified on peptide-Sepharose columns were incubated with radiolabeled CN and adsorbed to protein-A Sepharose. Bound CN was eluted by denaturing conditions.

This project has been terminated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00700-07 LPPS

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Ethanol Effects on Membrane-Bound Enzymes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	P.L. Hoffman	Section Chief	LPPS, NIAAA
	B. Tabakoff	Scientific Director	NIAAA
Others:	J.P. Whelan	Senior Staff Fellow	LPPS, NIAAA
	L. Karrberg	Special Volunteer	USP, NIAAA
	J. Contrera	Special Volunteer	USP, NIAAA
	E. Romm	Special Volunteer	USP, NIAAA
	D. Goldman	Section Chief	LCS, NIAAA

COOPERATING UNITS *(if any)*

Univ. of Minnesota, Minneapolis (J.A. Halikas); Washington Univ. Sch. Med., St. Louis (E. Devor); MDB, NIDDKD (A.M. Spiegel)

LAB/BRANCH

Laboratory of Physiologic & Pharmacologic Studies

SECTION

Section on Receptor Mechanisms

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Ave., Rockville, MD 20852

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.0	2.0	3.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

It has been postulated that the activities of certain enzymes may be markers of a genetic predisposition to alcoholism. We previously showed that low fluoride-stimulated platelet adenylate cyclase (AC) activity, and an increased sensitivity of platelet monoamine oxidase (MAO) to in vitro inhibition by ethanol, effectively discriminated alcoholic and non-alcoholic individuals. We have now examined the heritability of platelet fluoride-stimulated AC activity in families with alcoholic members, and have found familial transmission, with a major gene effect, for this enzyme activity. The major gene was transmitted as a Mendelian co-dominant. Transmission of basal AC activity was more complex. The findings are compatible with the possibility that fluoride-stimulated AC activity, which is transmitted as a single major gene in families of alcoholics, could represent a trait marker for a predisposition to alcoholism. In addition to $G_{s\alpha}$, future studies will quantitate AC with the use of antibodies raised against specific peptides. The studies described will help to determine whether the observed differences in platelet enzyme activities between alcoholics and non-alcoholics are genetically based, and may be a marker for a predisposition to alcoholism, or are a consequence of ethanol consumption.

This project has been terminated.

Publications:

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Tabakoff B, Whelan JP, Hoffman PL. Two biological markers of alcoholism. In: Banbury Report: molecular biology and genetics of alcoholism. Cold Spring Harbor Laboratory Press. 1990;195-204.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AA 00702-07 LPPS

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Ethanol Modification of Neurotransmitter Receptor-Effector Coupling

PRINCIPAL INVESTIGATOR *(List other professional parsonnel below the Principal Investigator.) (Neme, title, laboratory, and institute affiliation)*

P.I.:	P.L. Hoffman	Section Chief	LPPS, NIAAA
	B. Tabakoff	Scientific Director	NIAAA
Others:	P. Valverius	Visiting Associate	LPPS, NIAAA
	J.P. Whelan	Senior Staff Fellow	LPPS, NIAAA
	K.A. Grant	Staff Fellow	USP, NIAAA
	L. Snell	NRC Fellow	LPPS, NIAAA

COOPERATING UNITS *(if any)*
Metabolic Disease Branch, NIDDKD (A.M. Spiegel); IRCM, NHLBI (J. Moss); VA Medical Ctr., Portland, OR (J. Crabbe); BIOQUAL (B. Till, T. Calzone)

LAB/BRANCH
Laboratory of Physiologic & Pharmacologic Studies

SECTION
Section on Receptor Mechanisms

INSTITUTE AND LOCATION
NIAAA, 12501 Washington Ave., Rockville, MD 20852

TOTAL MAN-YEARS: 3.4	PROFESSIONAL: 3.4	OTHER: 0.0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Ethanol selectively alters the function of neurotransmitter and neuromodulator receptors in the CNS, and adaptations in receptor function may be associated with ethanol tolerance and/or physical dependence. Previous work showed decreased stimulation of adenylate cyclase by various agonists and by guanine nucleotides, and decreased high-affinity binding of a beta-adrenergic agonist and forskolin in certain brain regions of mice fed ethanol chronically. These results suggested a quantitative or qualitative change in the stimulatory guanine nucleotide binding protein, Gs. Quantitation of the two (46 and 52 kDa) forms of Gs-alpha, and of several other G-protein alpha and beta subunits, in various brain regions by Western blot analysis, however, revealed no significant change in the levels of these proteins in ethanol-fed mice. Differences in proportion of the two forms of Gs-alpha among brain regions were observed, and the 46 kDa form was found to be resistant to cholera toxin-catalyzed ADP-ribosylation. The data indicate that chronic ethanol ingestion may alter the properties rather than amount of Gs-alpha, and further characterization of the function of the two forms of the protein in ethanol-fed mice is warranted. The NMDA receptor system is also changed in ethanol-fed mice. As measured by membrane binding and autoradiographic studies of MK-801, a non-competitive antagonist at the NMDA receptor, this receptor is up-regulated in hippocampus and other brain areas of ethanol-fed mice. The time course of changes in binding parallels susceptibility to ethanol withdrawal seizures. In addition, mice bred to be prone to ethanol withdrawal seizures (WSP) have more hippocampal MK-801 binding sites than mice bred to be resistant to ethanol withdrawal seizures (WSR), both before and after chronic ethanol ingestion. These findings support a role for the NMDA receptor-gated channel in ethanol withdrawal. In preliminary studies, NMDA-sensitive glutamate binding was also increased in ethanol-fed mice, while strychnine-insensitive glycine binding was not, suggesting a change in the structure of function of NMDA receptor-gated channels.

This project has been terminated.

Publications:

Tabakoff B, Hoffman PL. Neurochemical effects of alcohol. In: Frances RF, Miller SI, eds. Clinical textbook of addictive disorders. New York: The Guilford Press, 1990;501-525.

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Hoffman PL, Rabe CS, VAlverius P, Grant KA, Tabakoff B. Genetic differences in the N-methyl-D-aspartate receptor: Effects of ethanol and phenyclidine. In: Harris RA, Crabbe JC, eds. The genetic basis of alcohol and drug actions. New York: Plenum Press, in press.

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Gulya K, Grant KA, Valverius P, Hoffman PL. Brain regional specificity and time-course of changes in the NMDA receptor-ionophore complex during ethanol withdrawal, Brain Res 1991;547:129-134.

Gonzales RA, Hoffman PL. Receptor-gated ion channels may be selective CNS targets for ethanol. TIPS 1991;12:1-3.

Valverius P, Crabbe JC, Hoffman PL, Tabakoff B. NMDA receptors in mice bred to be prone or resistant to ethanol withdrawal seizures. Eur J Pharmacol 1990;184:185-189.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AA 00703-07 LPPS

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Neurohypophyseal Peptides and Ethanol Tolerance

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	P.L. Hoffman	Section Chief	LPPS, NIAAA
Others:	L. Liu	Guest Researcher	USP, NIAAA
	J.R. Dave	Senior Staff Fellow	LPPS, NIAAA
	K. Gulya	Visiting Scientist	LPPS, NIAAA
	G. Szabo	Visiting Associate	LPPS, NIAAA
	K.A. Grant	Staff Fellow	USP, NIAAA

COOPERATING UNITS *(if any)*
VRB, DRS (C. Hansen); BIOQUAL (B. Till, T. Calzone)

LAB/BRANCH
Laboratory of Physiologic & Pharmacologic Studies

SECTION
Section on Receptor Mechanisms

INSTITUTE AND LOCATION
NIAAA, 12501 Washington Ave., Rockville, MD 20852

TOTAL MAN-YEARS: 3.9	PROFESSIONAL: 3.2	OTHER: 0.7

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Arginine vasopressin (AVP) and related peptides, when administered exogenously, prolong the duration of tolerance to ethanol by an action at CNS V-1 receptors. Previous work suggested that stimulation of c-*fos* expression in the septum by AVP contributes to this action, and current structure-activity studies of peptide (AVP(4-9), nerve growth factor) effects on ethanol tolerance support this hypothesis. The finding that a V-1 receptor antagonist enhanced the rate of loss of ethanol tolerance suggested that endogenous AVP is important for maintaining tolerance. Studies with rats bred to contain the diabetes insipidus (DI) gene (which do not produce functional vasopressin) were carried out to evaluate this postulate. Rats homozygous for the DI gene acquired functional ethanol tolerance, but lost it more rapidly than normal rats or those heterozygous for the DI gene. These data led us to examine AVP synthesis during chronic ethanol treatment. In both mice and rats, hypothalamic AVP mRNA was decreased by chronic ethanol exposure. The data, particularly in rats, suggested that the effect of ethanol was to uncouple vasopressin synthesis and secretion. *In situ* hybridization also revealed that dehydration increased, and chronic ethanol ingestion decreased, vasopressin mRNA in hypothalamic areas outside the SON and PVN and in the bed nucleus of the stria terminalis, an extrahypothalamic nucleus whose neurons project to the lateral septum. These results indicate that an increase in vasopressin synthesis in brain is not necessary in order for the hormone to maintain tolerance. Understanding the mechanism by which AVP influences tolerance to ethanol may lead to benign means for the manipulation of tolerance and, possibly, of ethanol intake.

This project has been terminated.

PHS 6040 (Rev. 1/84) GPO 914-918

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00705-05 LPPS

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
In Vitro Models for Ethanol Effects on Receptor-Mediated Processes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	P.L. Hoffman	Section Chief	LPPS, NIAAA
	B. Tabakoff	Scientific Director	NIAAA
Others:	K. Hellevuo	Visiting Fellow	USP, NIAAA

COOPERATING UNITS *(if any)*

None

LAB/BRANCH
Laboratory of Physiologic & Pharmacologic Studies

SECTION
Section on Receptor Mechanisms

INSTITUTE AND LOCATION
NIAAA, 12501 Washington Ae., Rockville, MD 20852

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A major focus of our work involves an evaluation of the acute and chronic effects of ethanol in the CNS. However, the brain represents a heterogeneous collection of cell types, and distinction of direct and indirect effects of ethanol can be difficult. In vitro cell culture systems can be used to monitor specific, direct effects of ethanol, for comparison and contrast with results obtained in brain tissue and in vivo. Current studies of the effect of ethanol on NMDA receptors in primary cultures of cerebellar granule cells are now described in project AA00706. In addition, investigations of serotonin (5HT-3) receptors in NCB20 somatic cell hybrids are being performed, and are based on the findings that 1) ethanol enhances the action of agonists at the 5HT-3 receptor in electrophysiological studies of NCB20 cells, and 2) 5HT-3 receptor antagonists block the discriminative stimulus properties of ethanol (see Project AA00707). To determine whether ethanol directly affects the interaction of ligands with the 5HT-3 receptor, antagonist and agonist binding to these receptors were assessed by filter binding techniques. The antagonist, ^{3}H-GR65630, bound to a single site on NCB20 membranes, and agonist (5HT, 2-Me-5HT) displacement curves were best fit by a one-site model. Ethanol (12.5 - 100 mM) had no significant effect on either agonist or antagonist binding, and may instead affect receptor-effector coupling processes. The behavioral studies suggest that the action of ethanol at the 5HT-3 receptor is important in mediating subjective effects of ethanol (e.g., reinforcement, intoxication) and studies in cultured cells may suggest a biochemical mechanism underlying these in vivo responses.

This project has been terminated.

Publications:

Hellevuo K, Hoffman PL, Tabakoff B. Ethanol fails to modify [3H]GR65630 binding to 5-HT-3 receptors in NCB-20 cells and in rat cerebral membranes. Alcoholism: Clin Exp Res, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AA 00483-02 LPPS

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Cloning, Expression and Site-directed Mutagenesis of G Protein-Linked Receptors

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	C.M. Fraser	Section Chief	LPPS, NIAAA
Others:	N.H. Lee	IRTA Fellow	LPPS, NIAAA
	T.M. Savarese	Special Volunteer	LPPS, NIAAA
	C-D. Wang	Visiting Associate	LPPS, NIAAA

COOPERATING UNITS *(if any)*
NINDS, NIH (J.C. Venter); Univ. Geneva (J-P. Giacobino); Israel Inst. for Biological Res (A. Fisher)

LAB/BRANCH
Laboratory of Physiologic and Pharmacologic Studies

SECTION
Section on Molecular Neurobiology

INSTITUTE AND LOCATION
NIAAA, 12501 Washington Ave., Rockville, MD 20852

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.1	2.8	0.3

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Neurotransmitter receptors known to mediate their cellular actions through guanine nucleotide regulatory binding proteins (G proteins) comprise a large and diverse gene superfamily and modulate the activity of a variety of important physiological functions. Ethanol has been shown to specifically affect G protein-mediated signal transduction and this finding suggests that changes in G protein-linked systems may be involved in the manifestations of chronic ethanol ingestion. In order to better understand the molecular mechanisms of signal transduction mediated by this family of receptors we have utilized a number of molecular biological approaches including gene cloning, permanent receptor gene expression in cultured cells, and site-directed mutagenesis to study the relationship between receptor structure and function and the mechanisms of receptor regulation. CHO cell lines permanently transfected with a single muscarinic acetylcholine receptor subtype are proving to be of great utility in the characterization of muscarinic agonists with M1 subtype selectivity. These agents are considered as candidate drugs for the treatment of Alzheimer's disease. The ability to modify the amino acid sequence of a receptor using site-directed mutagenesis has allowed for the identification of conserved cysteine residues in muscarinic acetylcholine receptors that are involved in ligand binding and receptor activation by agonists. Also, we have demonstrated that beta-adrenergic and muscarinic acetylcholine receptors expressed in the same cell line are down-regulated in a coordinated fashion by agonists. Since both of these receptor subtypes can be found on peripheral tissues and in the central nervous system, the phenomenon of cross-regulation becomes an important area of study. Lastly, clones encoding a fat cell specific beta-adrenergic receptor (beta3A-subtype) were isolated from a rat brown adipose tissue cDNA library. Expression of this receptor, which is most likely involved in mediated lipolysis in adipose tissue is reduced 60-70% in obese (fa/fa) Zucker rats as compared with lean controls, suggesting that decreased levels of this receptor may play a role in obesity.

Previous title: Molecular Biology of G Protein Coupled Receptors

Project Description:

Investigators:

C.M. Fraser	Section Chief	LPPS, NIAAA
A. Fisher	Department Head	Israel Inst. for Biomedical Res.
J-P. Giacobino	Professor	Univ. Geneva
H. Higgins	Summer Student	LPPS, NIAAA
N.H. Lee	IRTA Fellow	LPPS, NIAAA
T. Savarese	Special Volunteer	LPPS, NIAAA
J.C. Venter	Section Chief	LPPS, NIAAA
C-D. Wang	Visiting Associate	LPPS, NIAAA

Objectives:

A majority of all hormones and neurotransmitters generate intracellular signals through a process that involves binding to a cell surface receptor that couples to one or more guanine nucleotide regulatory proteins (G protein) and modulates the activity of several intracellular effector systems. The molecular mechanisms involved in G protein-mediated signal transduction have been largely unknown. Recent advances in the application of molecular biology to the study of G protein-linked receptors has provided the first information on the primary and secondary structures of these molecules. The hallmark of all G protein-linked receptors is the presence of seven transmembrane alpha-helices which display considerable amino acid homology. Our laboratory has cloned and sequenced several receptors from this family and we are now utilizing techniques including permanent gene expression in cultured cells and site-directed mutagenesis to explore the relationship between receptor structure and function in order to elucidate the molecular basis for such functions as ligand binding, receptor activation by agonists and receptor desensitization. Identification of the mechanisms involved in G protein-mediated signal transduction will provide a starting point for the more rational design of therapeutic agents targeted to these receptors.

Methods Employed:

Neurotransmitter receptor genes were inserted into plasmid expression vectors containing selectable markers (such as aminoglycoside phosphotransferase) and these expression vectors were used to transfect cultured cells lacking endogenous receptors following standard published protocols. Two days after transfection, cells were placed into selective medium and only those cells having stably integrated plasmid DNA into their genome survive. Colonies derived from single cells were isolated, expanded and assayed for the presence of receptors using radioligand binding assays. For construction of mutated receptors, the coding region of a receptor gene was cloned into bacteriophage M13 and single base mutations that direct the substitution of one amino acid for another in the expressed protein were introduced by oligonucleotide-directed mutagenesis according to the method of Kunkel (1985). Authenticity of mutations was confirmed by dideoxy sequencing and mutant receptor genes were expressed in cultured cells using plasmid expression vectors. The function of each mutant receptor was analyzed by ligand binding experiments and in biochemical assays of cAMP accumulation, polyphosphoinositide hydrolysis, arachidonic acid metabolism and/or [3H]-thymidine incorporation.

Cloning of cDNAs from phage libraries was done using plaque hybridization to [32P]-labeled cDNA probes encoding rat beta1- and beta2-adrenergic receptors under conditions of low stringency. Positive phage DNA was subcloned into M13 for template preparation for automated DNA sequencing. Analysis of DNA for sequence homologies was done using the Suite software from IntelliGenetics.

RNA isolation was performed using extraction with guanidine isothiocyanate; poly (AO+ RNA was obtained by oligo (dT) chromatography. Poly (A)+ RNA was electrophoresed in agarose-formaldehyde gels and transferred to membranes for Northern blot analysis.

Major Findings:

1. Identification of amino acids involved in ligand binding and receptor activation by agonists in m1 muscarinic receptors using site-directed mutagenesis and permanent gene expression.

The overall objective of this project is to identify the specific amino acids that are involved in m1 muscarinic acetylcholine (m1 mAChR) function, especially ligand binding and receptor activation. There are nine cysteine residues within the rat m1 muscarinic acetylcholine receptor that are conserved in all muscarinic subtypes; over the past years Drs. Savarese and Wang have converted each of these residues to serine, and have expressed these mutant receptor genes in CHO cells which lack muscarinic receptors. The ability of these mutant receptors to bind muscarinic ligands and to activate the phospholipase C-dependent hydrolysis of membrane phosphoinositides (PI) was then assessed. The major findings of these studies have been summarized in last year's report. In the current year we are completing the characterization of some of these Cys-to-Ser mutant receptors. For example, we have found that none of the m1 mAChRs, either wild type or mutants, undergo a significant rightward shift in carbachol binding in the presence of GTP.

One of the unresolved issues concerns the fact that membranes from CHO cells transfected with either the Ser98 or Ser178 mutant receptor genes displayed negligible binding of the muscarinic antagonists [3H]-quinuclidinyl benzilate ([3H]-QNB). Biochemical evidence from Hulme's laboratory has indicated that in the native receptor, the cysteines at positions 98 and 178, which reside in the putative extracellular loops of the receptor between transmembrane regions II and III, and IV and V, respectively, are involved in a disulfide linkage. Our data suggested that either 1) the affinity of the receptor for [3H]-QNB binding is greatly reduced by the loss of this postulated disulfide bond, 2) that in the absence of such a bond, the receptor does not undergo proper folding, processing and/or insertion into the membrane or 3) these mutant genes do not undergo transcription in CHO cells. In the past year we have attempted to sort through these possibilities. Using RNA hybridization analysis, we have identified a number of CHO cell clones that had been transfected with either the Ser98 or Ser178 mutant receptor genes that express 2.0 kb m1 mAChR mRNA transcripts. Membranes from these clones do not bind

[3H]-QNB, and the intact cells do not activated PI hydrolysis even in the presence of 20 mM carbachol. However, it is not known if the receptor against peptides representing portions of the extracellular domains of the rat m1 mAChR to immunoprecipitate solubilized m1 AChRs. By labelling the proteins with 35S-immunoprecipitate on SDS-PAGE gels, it may be possible to identify and quantitate the levels of m1 mAChR receptor protein in CHO cells expressing the mutant and wild type receptors. This same technique could be used to study any post-translational modification of the m1 mAChR, such as phosphorylation or palmitoylation.

Our previous studies had demonstrated that conversion of Cys394 to serine yields a receptor that when expressed in CHO cells has a considerably reduced ability to activate PI hydrolysis in response to carbachol. Since this cysteine resides on the extracellular loop between transmembrane regions VI and VII and is in close proximity to another cysteine residue, Cys391, it is possible that these cysteines may also form a disulfide linkage. However, we found that the Ser391 mutant receptor did not display a reduction in its ability to activate PI turnover relative to that in cells expressing a wild type receptor at the same receptor density. This suggested that Cys391 and Cys394 are not linked via a disulfide bond to each other, but does not rule out the possibility that they are individually linked to other, nonconserved cysteines within the m1 mAChR.

2. Selective agonist profile of AF102B in cells expressing various muscarinic acetylcholine receptor subtypes.

AF102B, a highly rigid cholinergic agonist with M1 subtype selectivity has been suggested as a promising treatment approach for Alzheimer's disease based on its unique neurochemical, electrophysiological and psychopharmacological profile. In collaboration with Dr. Abraham Fisher from the Israel Institute for Biological Research, the ligand binding properties and biochemical responses of AF102B were assayed in CHO cells transfected with either m1 or m3 muscarinic acetylcholine receptor (m-AChR) subtypes and in PC-12 cells expressing primarily the m4 m-AChR subtype. Like carbamylcholine, a m-AChR agonist, AF102B stimulated hydrolysis of phosphoinositides (PI) in CHO cells transfected with the m1 m-AChR; this response was sensitive to atropine. Unlike, carbamylcholine, AF102B failed to stimulate PI hydrolysis in CHO cells transfected with the m3 m-AChR, or in SK-N-SH human neuroblastoma cells (expressing mostly m3 m-AChR). Forskolin-stimulated adenylate cyclase (AC) activity in PC-12 cells was partially attenuated by AF102B and this effect was blocked by atropine. These data suggest that AF102B selectively activates m1 and m4 m-AChR, but no m3 m-AChR subtypes.

3. Cross-regulation of the m1 muscarinic acetylcholine receptor upon activation of the beta2-adrenergic receptor-adenylate cyclase pathway.

Muscarinic acetylcholine and beta-adrenergic receptors are members of a superfamily of receptors that couple to guanine nucleotide binding proteins (G-proteins). At present, five distinct muscarinic acetylcholine receptors have been cloned from various mammalian species and are designated m1-m5. Agonist activation of the m1, m3 and m5 muscarinic receptor subtypes results in the hydrolysis of phosphoinositides; while, activation of the m2 and m4 subtypes are linked to an inhibition of forskolin-stimulated adenylate cyclase activity. Beta-adrenergic receptors are believed to be comprised of three subtypes, designated beta1- beta3, and activation of these G-protein coupled receptors leads to stimulation of the adenylate cyclase pathway. While great strides have been made in understanding the molecular basis for such functions as ligand binding and receptor activation by agonists, less is known on the phenomenon of cross-regulation (cross-talk) of G-protein coupled receptors and the molecular mechanisms involved. Since both the muscarinic acetylcholine and beta-adrenergic receptors can be found on peripheral tissues and the central nervous system, the potential for cross-regulation becomes an important new area for research.

Using established molecular biological techniques, we have permanently co-expressed the genes for both the m1 muscarinic acetylcholine receptor (m1-mAChR) and beta2-adrenergic receptor (beta2-AR) in CHO cells. A cell line (MB-CHO) was established that expressed comparable levels of m1-mAChR and beta2-AR (1 pmol receptor/mg protein). In work by Dr. Lee and Ms. Higgins, the muscarinic agonist carbamylcholine was shown to stimulate PI hydrolysis in transfected MB-CHO cells with an EC50 of 10 mM. Persistent activation of the m1-mAChR resulted in a time- and concentration-dependent decrease in m1-mAChR but not beta2-AR. As much as 65% of the m1-mAChR was down-regulated over a 24 hour period with 1 mM carbamylcholine. A corresponding decrease by about 30-40% in the steady state level of m1-mAChR mRNA was seen during this time. Isoproterenol, a beta2-adrenergic receptor agonist, stimulated adenylate cyclase activity in MB-CHO cells with an EC50 of 10 nM. Activation of the beta2-AR with 2 mM isoproterenol for 24 hours decreased the level of beta2-AR and beta2-AR mRNA by 70% and 40%, respectively. Furthermore, stimulation of the beta2-AR-adenylate cyclase pathway leads to a time- and concentration-dependent decrease in the levels of m1-mAChR and m1-mAChR mRNA. Isoproterenol, 2 mM, produced a time-dependent decrease in the level of m1-mAChR from 2 to 12 hours with maximal decreases (50%) occurring after 12 hours. A corresponding decrease in efficacy of carbamylcholine to stimulate PI hydrolysis was observed. Steady state levels of m1-mAChR mRNA were decreased after only 6 hours of isoproterenol pretreatment, while maximal decreases in mRNA levels occurred between 12 and 18 hours and returned to control levels by 24 hours. The isoproterenol induced decrease in m1-mAChR was blocked by either propranolol (beta-AR antagonist) or H-8 (A-kinase inhibitor), and mimicked by forskolin and dibutyryl cAMP. These data establish unidirectional cross-regulation of the m1-mAChR and m1-mAChR mRNA involving the beta2-AR-adenylate cyclase pathway and A-kinase in a transfected cell line.

The molecular mechanisms responsible for m1-mAChR and beta2-AR cross-regulation are currently under investigation. For example, studies on the effect of beta2-AR activation on m1-mARhR mRNA stability have been initiated. In addition, we have identified a potential recognition sequence motif for A-kinase (a serine residue preceded by several basic amino acids) on the third intracellular loop of the m1-mAChR. The signal for down-regulation of the m1-mAChR by beta2-AR activation may reside in this motif. Hence, site-directed mutagenesis experiments with the m1-mAChR will be utilized to address this issue.

4. Cloning and expression of a fat cell-specific beta-adrenergic receptor subtype.

Brown adipose tissue is the main effector of cold- and diet-induced thermogenesis in rodents, a process that can represent a major expenditure of energy and play an important role in overall energy balance. Because brown adipose tissue has been demonstrated in humans of all ages and is often atrophied and quiescent in obese animals, much interest has recently been directed toward development of compounds that stimulate this metabolic response as possible anti-obesity agents. In collaboration with Dr. Jean-Paul Giacobino from the University of Geneva and Dr. J. Craig Venter from NINDS, clones encoding a fat cell-specific beta-adrenergic receptor (termed a beta3a-subtype) were isolated from a rat brown adipose tissue cDNA library. This receptor is a single polypeptide of 400 amino acids and displays 49%, 40% and 80% identity respectively with rat beta1 and beta2-adrenergic and human beta3-adrenergic receptors. Work in our laboratory demonstrated that the fat cell beta-receptor expressed in CHO cells displays high affinity for BRL 37344 (Ki = 250 nM) and low affinity for classical beta-adrenergic antagonists such as propranolol (Ki = 1.5 uM). The order of agonist potency for accumulation of intracellular cAMP in transfected cells correlates with that for stimulation of lipolysis in brown adipocytes (BRL 37344 > isoproterenol > norepinephrine > epinephrine). The beta3A-adrenergic receptor is preferentially expressed in brown and white adipose tissue in rats and humans where it represents the predominant beta-receptor subtype. The beta3A-adrenergic receptor mRNA in brown and white adipose tissue of obese (fa/fa) Zucker rats is reduced 60-70% as compared with lean controls suggesting that decreased expression of this unique receptor subtype may play a role in obesity.

Significance to Biomedical Research and the Program of the Institute:

The results obtained in these projects further our understanding of the molecular mechanisms involve in G protein-mediated signal transduction. As ethanol has been demonstrated to effect the activity of several important elements involved in G protein-mediated signal transduction, these results provide a basis for testing hypotheses regarding the locus of ethanol's actions in these processes. The data that are forthcoming from the site-directed mutagenesis studies will be useful in mapping the similarities and differences in the ligand binding sites of various G protein-linked receptors. Since these receptors are targets for pharmacological intervention in a large number of diseases this information may be potentially useful in the design of new pharmaceutical agents with increased specificity for a particular receptor subtype.

Proposed Course:

Site-directed mutagenesis experiments with m1 muscarinic and alpha2-adrenergic receptors are continuing, and based on known structure-activity relationships for adrenergic and muscarinic ligands we are investigating the role of other amino acids as potential sites for receptor-ligand interactions. The role of putative sites within the m1 muscarinic receptor for phosphorylation by protein kinases A and C are also being investigated for their role in homologous and heterologous receptor down-regulation. These studies are being extended to include the m2 muscarinic acetylcholine receptor subtype to address whether the same mechanism(s) are involved in down-regulation of a muscarinic receptor that preferentially inhibits adenylate cyclase.

Studies are also currently underway to utilize mutagenesis techniques to identify the domain(s) within the beta3A-adrenergic receptor that are responsible for the unique ligand binding properties of this receptor subtype as compared with beta2- and beta2-receptors. These studies will help to elucidate the regions of the receptor that directly participate in and indirectly influence ligand binding and may ultimately be exploited in the design of subtype-specific reagents. Methods are being developed to allow for quantitation of this receptor subtype by radioligand binding assays. Also, the potential role of the beta3A-adrenergic receptor in human obesity is being investigated.

Publications:

Buck MA, Fraser CM. Muscarinic acetylcholine receptor subtypes which selectively couple to phospholipase C: pharmacological and biochemical properties, Biochem Biophys Res Comm 1990;173:666-72.

Fraser CM. Molecular biology of adrenergic receptors: Model systems for the study of G protein-mediated signal transduction, Blood Vessels 1991;28:93-102.

Fraser CM. The molecular basis of G protein-coupled receptor function. Proceedings of the Lovelace Medical Foundation Annual Scientific Conference: Signaling Mechanisms in Secretory and Immune Cells, in press.

Revelli J-P, Pescini R, Muzzin P, Seydoux J, FitzGerald MG, Fraser CM, Giacobino J-P. Changes in beta1 and beta2-adrenergic receptor mRNA levels in brown adipose tissue and heart of hypothyroid rats, Biochem J, in press.

Wang C-D, Buck MA, Fraser CM. Alpha2-adrenergic receptors: Identification of amino acids involved in ligand binding and receptor activation by agonists, Mol Pharmacol, in press.

Savarese TM, Fraser CM. In vitro mutagenesis and the search for structure-function relationships among G protein-linked receptors, Biochem Pharmacol, in press.

Fraser CM. Expression of receptor genes in cultured cells. In: Hulme EC, ed. Receptors: A practical approach. London: IRL Press, 1990;263-76.

Savarese TM, Venter JC, Fraser CM. In vitro-mutagenesis and the search for structure-function relationships among G protein-linked receptors, Biochem J 1990, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00484-02 LPPS

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Structure/Function Analysis of the GABAA Receptor

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	C.M. Fraser	Section Chief	LPPS, NIAAA
Others:	A.L. Buller	IRTA Fellow	LPPS, NIAAA
	G.A. Hastings	IRTA Fellow	LPPS, NIAAA
	E.F. Kirkness	Visiting Associate	LPPS, NIAAA

COOPERATING UNITS *(if any)*

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SECTION
Section on Molecular Neurobiology

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TOTAL MAN-YEARS: 1.9
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OTHER:

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☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

GABA is the major inhibitory neurotransmitter in the brain. A variety of compounds, including benzodiazepines, barbiturates and ethanol potentiate GABA responses. In order to better understand the molecular basis of ligand-gated ion channel function and to identify the sites within the GABA receptor at which ethanol may act, we have established systems for the expression of cloned receptor-channel complexes in Xenopus oocytes injected with receptor-specific mRNA and in cultured cells transfected with receptor-specific cDNAs. Molecular cloning of the GABAA receptor has confirmed the existence of at least six alpha, three beta, two gamma, one delta, and one rho subunits. The stoichiometry of these subunits in the native receptor is not known; neither are the structural determinants that influence ligand binding or maintain the chloride ion selectivity of the channel. We have undertaken a series of studies to determine the role of glycosylation in the functional expression of the GABAA receptor. Incubation of Xenopus oocytes microinjected with alpha1, beta1 and gamma2 subunit-specific mRNAs with tunicamycin inhibits the expression of the GABA receptor. Site-directed mutagenesis is being used to determine the contribution of each putative glycosylation site to functional receptor expression. These studies will determine which glycosylation sites are required for functional assembly of the GABA receptor and whether glycosylation influences the binding of ligands. We have also begun a characterization of the amino acids on the alpha subunit of the GABA receptor that influence the binding of benzodiazepines. We have chosen the alpha6 subunit as a model because the presence of this subunit in a functional receptor complex is associated with high affinity binding for the benzodiazepine, RO15-4513 (a putative alcohol antagonist), but not for other benzodiazepines or beta-carbolines. Site-directed mutagenesis has been used to construct mutations in the alpha1 and alpha6 subunits in a region previously determined to be required for benzodiazepine binding. The properties of the mutant GABA receptors are being assessed in Xenopus oocytes and cultured cells.

Previous title: Expression of ligand-gated ion channels

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description:

Investigators:

C.M. Fraser	Section Chief	LPPS, NIAAA
A.L. Buller	IRTA Fellow	LPPS, NIAAA
G.A. Hastings	IRTA Fellow	LPPS, NIAAA
E.F. Kirkness	Visiting Associate	LPPS, NIAAA

Objectives:

Based on functional diversity, the existence of different subtypes of GABAA receptors has been proposed. The GABAA receptor has been cloned and sequenced from several mammalian sources and it is composed of multiple subunits that display considerable amino acid homology. GABAA receptor subunits exhibit unique distributions throughout the nervous system suggesting that the observed pharmacological diversity and the differential sensitivity of GABA receptors from various brain regions to the effects of ethanol may, in part, result from a unique combination of receptor subunits. In order to address the question of the molecular basis of GABA receptor function, we have established system for the expression of receptor subunits in Xenopus oocytes and cultured human embryonic kidney cells (293 line) that will allow for study of the electrophysiological properties of individual receptor subunits an/or combinations of receptor subunits. Mutagenesis techniques will be used to identify the amino acid sequences that play a role in high affinity benzodiazepine binding, post-translational receptor modifications, receptor desensitization, ion selectivity of the channel and ethanol modulation of receptor function.

Methods Employed:

A functional GABAA receptor requires the coexpression of alpha, beta, and gamma subunits. It was necessary to clone several subunit cDNAs so that expression in Xenopus oocytes and mammalian kidney cells could be measured. We have cloned and sequenced the rat alpha6 and gamma2 subunits from a whole brain cDNA library. Partial length clones were obtained in both cases. The gamma2 cDNA clone required the addition of 200 hundred base pairs of 5' DNA which was accomplished by PCR amplification of first strand brain cDNA of a region bounded by unique restriction sites. The PCR product and gamma2 cDNA were digested with these enzymes and ligated together resulting in a full length clone. The same procedure was used to add the 3' end to the alpha6 cDNA clone.

The alpha1 (provided by Michel Krestchatisky and Allan Tobin) and alpha6 cDNAs were subcloned into the plasmid pSELECT (Promega) for in vitro mutagenesis. Mutagenic primers were designed that contain the desired mutations. These primers were annealed to single-stranded DNA in separate reactions. T4 DNA polymerase was used to create double-stranded DNA which was introduced into E. coli. Plasmid DNA from selected clones was sequenced to verify that the mutations were created. These mutated and wild-type alpha1, alpha6, beta1, and gamma2 cDNAs were subcloned into the mammalian expression vector pCDM8 (Invitrogen).

Poly (A+) RNA was transcribed in vitro from cloned cDNAs encoding specific GABAA receptor subunits. Stage V or VI oocytes are harvested from female Xenopus laevis anesthetized with Tricaine and were dissociated from ovarian sacs by mechanical and enzymatic treatment. Oocytes were suspended in ND-96 solution and incubated at 18° C for 12-18 hours prior to injection. Each oocyte was microinjected with 10-100 ng poly (A+). Membrane ion currents were measured within 48-72 hours following injection using the whole-cell patch-clamp technique. Appropriate receptor agonists and antagonists were administered from a pipette placed near the oocyte. Data were digitized and stored on magnetic media for subsequent analysis.

Human embryonic kidney (293) cells were transfected with plasmid expression vectors containing the appropriate GABA receptor subunits using a standard calcium phosphate precipitation protocol. Cells were harvested 48-72 hours following transfection for assay using the GABA receptor specific radioligands.

Major Findings:

We have expressed functional GABAA receptors in Xenopus oocytes microinjected with RNA coding for the alpha1, beta1 and gamma2 subunits. Bath application of GABA elicits inward chloride currents in voltage clamped oocytes with an EC50 of approximately 10 uM and a Hill co-efficient if 1.25. GABA currents are potentiated by phenobarbital (200% of control) and by flunitrazepam (35% of control). Benzodiazepine potentiation is blocked by the benzodiazepine antagonist, RO15-1788. Sequence analysis of the alpha1 subunit has revealed that this polypeptide contains two consensus sequences for N-linked glycosylation (Asn-X-Ser/Thr). In studies by Dr. Buller, tunicamycin was used to investigate the role of N-linked glycosylation in expression of the GABA receptor in Xenopus oocytes. Tunicamycin inhibits the transfer of the oligosaccharide precursor to the growing polypeptide chain and thus prevents the formation of all forms of N-linked glycosylation. Oocytes were injected with GABA receptor subunit-specific RNAs mixed in equimolar stoichiometry alone or in combination with tunicamycin (25 mg/ml) and incubated for 48 hours. Tunicamycin completely abolished functional expression of the GABA receptor. To further investigate the role of glycosylation in expression of the GABA receptor, glycosylation-deficient mutants have been generated containing only one of the two sites for glycosylation (at positions 10 and 110) or missing both sites. We are currently investigating the effects of these mutations on expression of the GABA receptor in Xenopus oocytes and the pharmacology of the receptors expressed in 293 cells.

Expression and characterization of the alpha1/alpha6 chimaeric GABA receptors are in progress by Dr. Hastings.

Significance to Biomedical Research and the Program of the Institute:

The GABAA receptor is a hetero-oligomeric complex that interacts with GABA depressant drugs (benzodiazepines, barbiturates, and ethanol) and produces inhibition of neural activity in the brain. The molecular basis for GABA receptor function is not well understood. In situ localization of GABAA subunit mRNAs indicates a complex pattern of expression in brain regions. The studies underway in this project are directed toward a characterization of the sites within the GABA receptor-chloride channel complex that are associated with known functional activities. This information will be critical in elucidating the mechanisms of alcohol modulation of receptor function. Identification of the amino acids or domains within the alpha6 subunit that are responsible for conferring high affinity binding of RO15-4513 may help to elucidate how this agent antagonizes some of the behavioral effects of alcohol.

Proposed Course:

The six chimeric alpha1/6 mutants will be transfected into 293 kidney cells along with wild-type beta1 and gamma2 cDNAs. Binding of Ro15-4513 and other benzodiazepines to membranes from each mutant will be measured and compared with wild-type alpha1 and gamma2 cDNAs. Mutants with pharmacology approaching alpha6 will be targeted for further mutagenesis since each mutant contains 2-5 amino acid changes. In vitro transcribed RNA from the same cDNAs will be microinjected into oocytes to assess any effects on chloride channel function. The ability of ethanol to potentiate GABA-induced currents in these microinjected oocytes will also be determined.

The role of glycosylation in the functional expression of the GABAA receptor will be studied further by characterization of the properties of glycosylation-deficient receptor mutants in Xenopus oocytes and 293 cells. Additional mutations at putative sites of glycosylation in beta1 and gamma2 subunits will also be constructed and assayed.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00485-02 LPPS

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Gene Structure of GABA-A Receptor Subunits

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	E.F. Kirkness	Visiting Associate	LPPS, NIAAA
Others:	C.M. Fraser	Section Chief	LPPS, NIAAA
	G.A. Hastings	IRTA Fellow	LPPS, NIAAA
	T.L. Miller	Biologist	LPPS, NIAAA

COOPERATING UNITS *(if any)*
Laboratory of Clinical Studies, NIAAA (D. Goldman); Program Resources Inc., NCI-Frederick Cancer Research (M. Dean); Division of Genetics, Children's Hospital, Boston, MA (M. Lalande)

LAB/BRANCH
Laboratory of Physiologic and Pharmacologic Studies

SECTION
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  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The major class of inhibitory neurotransmitter receptors, the GABAA receptors, are modulated by a range of depressant drugs (eg benzodiazepines, ethanol) and their dysfunction has been associated with several neurological disorders. Recently, cDNA cloning has revealed the existence of numerous receptor subunit types. However, in the absence of genomic clones, it has been difficult to establish any link between inappropriate receptor expression and the implicated disease states. In addition, nothing is known of how expression of subunit genes is regulated in a coordinated and localized manner. Studies on the intron/exon structure, DNA sequence and chromosomal localization of the gene encoding the human beta2 subunit have been completed. Repetitive DNA sequences within intronic segments of the gene were used to detect a polymorphic region of the gene. This polymorphism can now be used to screen for any linkage between the beta1 gene and known genetic disorders. These types of studies are being extended to include other GABA receptor genes, in particular those encoding the alpha6 and gamma2 subunits. We have obtained six independent putative gamma2 clones from a human genomic library in our initial screen. We will map the positions of restriction sites and gamma2 introns/exons on these clones. Polymorphisms will be identified by probing these clones with oligonucleotides composed of known polymorphic repeated sequences and by restriction fragment length mapping of human genomic DNA. We will also determine the chromosomal position of these subunit genes by in situ hybridization. Cloned fragments of the human beta3 subunit gene were used to map this gene to the Angelman/Prader-Willi region of human chromosome 15. It is possible that this gene may play a role in the pathogenesis of one or both of these syndromes. As the syndromes appear to result from a genetic imprinting process, an understanding of how expression of the beta3 gene is regulated may be of particular importance. Ongoing studies aim to define the regulatory elements of the beta3 gene using transfected PC12 cells and PC12 nuclear extracts.

Project Description:

Investigators:

E.F. Kirkness	Visiting Associate	LPPS, NIAAA
M. Dean	Staff Scientist	PRI, Inc.
C.M. Fraser	Section Chief	LPPS, NIAAA
D. Goldman	Section Chief	LPPS, NIAAA
G.A. Hastings	IRTA Fellow	LPPS, NIAAA
M. Lalande	Professor	Children's Hospital
T.L. Miller	Biologist	LPPS, NIAAA

Objectives:

The initial objective of this project is to clone and sequence genes that encode human GABAA receptor subunits. Subsequently, these clones will be used:

a) to probe for restriction fragment length polymorphisms (RFLPs) in families that exhibit inherited neurological disorders (eg alcoholism, schizophrenia);
b) to characterize the upstream regions of the genes, in order to determine how coordinated expression of receptor subunits is regulated in normal and diseased states.

Methods Employed:

A bovine cDNA, encoding the GABAA receptor beta1 subunit, was used to generate DNA probes by the polymerase chain reaction (PCR). Three probes, that together covered the whole protein-coding regions of the gene, were used successively to screen libraries of human genomic clones. Positive clones were rescreened with oligonucleotide probes that recognized particular exons specifically. DNA was prepared from the clones of interest and was mapped with several restriction enzymes. Fragments of the cloned DNA that contained beta1 exons were detected by Southern blotting and were subcloned for sequencing. Large fragments were subjected to a process of ordered deletions prior to sequencing. All sequencing was performed on automated instruments, using a fluorescent detection system.

A rat gamma2 cDNA restriction fragment was used to probe a human genomic DNA library. Six clones were isolated and subjected to restriction enzyme analysis. The pattern of restriction fragments indicates that each clone contains a unique genomic insert with two clones sharing an identical SacI band. The size of each genomic insert ranges from 13 to 18 kilobases.

In order to characterize and identify polymorphisms in the beta1 subunit of the GABA receptor, a portion of the intron between exons 8 and 9 that contained a tetranucleotide repeat sequence (GATA) was amplified from human genomic DNA using PCR. The PCR products were separated on a sequencing gel and the alleles were identified.

Major Findings:

The GABAA receptors belong to a superfamily of ligand-gated ion channels, which also includes nicotinic acetylcholine (nACh) and glycine receptors. Of these, only nACh studies on the intron/exon structure, DNA sequence and chromosomal localization of the gene encoding the human beta1 subunit have been completed. By examining DNA sequences within the introns of the beta1 subunit we found, in collaboration with Drs. M. Dean and D. Goldman, a tetranucleotide repeat sequence (GATA). Amplification of this region using the polymerase chain reaction revealed 7 alleles and a high degree of polymorphism in human populations. This polymorphism can now be used to screen for any linkage between the beta1 gene and known genetic disorders.

Studies by Dr. Kirkness on the DNA sequences of the beta1 gene, upstream of the protein-coding exons, indicate a complex organization. Using PCR, several cDNA clones have been identified that contain non-coding exons, upstream of the initiation ATG. Sequencing of these cDNAs has revealed at least two distinct non-coding exons that appear to be utilized in human brain. Ongoing studies aim to identify the tissue distribution of these different exons and their effects on the expression of the beta1 subunit protein.

In collaboration with Dr. Lalande, cloned fragments of the beta3 gene were used to map this gene to the Angelman/Prader-Willi region of human chromosome 15. At present, this is the only functional gene that is known to localize to the affected region. It may therefore play a role in the pathogenesis of one or both of these syndromes. As the syndromes appear to result from a genetic imprinting process, an understanding of how expression of the beta3 gene is regulated may be of particular importance. Genomic DNA containing the first three coding exons, and at least 10kb of sequence upstream of the initiation ATG, have been cloned from rat and human libraries. Intron/exon structure in this region of the beta3 genes is identical to that of the human beta1 gene. Sequencing of the DNA has revealed particularly high conservation of sequence between rat and human genes, even in non-coding regions. It is likely that the primary transcripts from the gene exhibit a conserved secondary structure, which may contribute to the regulated expression of the beta3 subunit. Using the human genomic clones as probes, polymorphic regions of the gene have been detected by RFLP analyses. Screening of Angelman families is currently in progress.

In order to analyse the promoter region of the gene, it is of benefit to have a cell line that expresses the gene normally. Several cell lines were examined, using PCR- analysis of reverse-transcribed RNA. One of these, the rat PC12 cell line, was found to contain beta3 subunit RNA. Ongoing studies aim to define the regulatory elements of the beta3 gene using transfected PC12 cells and PC12 nuclear extracts.

An assay has been developed that can detect the expression of GABA receptor subunits in small samples of tissue. Using PCR to amplify segments of reverse-transcribed RNA, the expression of all known subtypes of GABAA receptor subunits can be detected, using less than 1 ug of crude RNA derived from rat or mouse tissues. The assay has been used to compare patterns of subunit expression in the brains of long-sleep and short-sleep mice (which differ in their sensitivity to aspects of alcohol intoxication). All of the known subunits (alpha1-6, beta1-3, gamma1-2, delta) were found to be expressed in both mouse strains.

Currently, the assay is being used to identify which of the receptor subunits are expressed in specific tissue regions (eg adrenal medulla). This work aims to correlate subunit composition with specific physiological and pharmacological properties of the receptor.

Significance to Biomedical Research and the Program of the Institute:

GABAA receptors are the major inhibitory neurotransmitter receptors in mammalian brain. They represent the sites of action for an important class of drugs, the anxiolytic benzodiazepines. They are also likely to mediate at least some of the effects of several other depressant agents (eg barbiturates and ethanol). Owing to their central role in the process of neuronal inhibition and their association with anxiety and sedation, it would be predicted that dysfunction of receptor genes may likely result in some forms of neurological disorder. The probes derived from these human GABAA receptor subunit genes will be useful tools to screen for an association between the receptors and inherited neurological disease. In comparison with the few cDNA probes that are available at present, these probes will display a greater specificity for individual subunit subtypes, and are more likely to detect RFLPs that reside far from exons.

Little is known of how genes that encode neurotransmitter receptor subunits are regulated, in terms of their coordinated expression in specific brain regions. As has been demonstrated in other systems, disturbances in the mechanism of transcriptional regulation can be as critical as major defects in the exons of genes. The cloning and sequencing of upstream regions of genes encoding GABAA receptor subunits provides the first step to understanding these questions of transcriptional regulation.

Proposed Course:

1. Fragments of the isolated GABA receptor subunit genes will be used to screen for RFLPs and/or repeat polymorphisms in families that exhibit inherited neurological disorders (eg alcoholism, schizophrenia, manic depression).

2. The upstream regions of the genes will be examined in order to characterize the sequences and binding factors that determine how expression of the receptor is controlled.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AA 00400-06 USP

PERIOD COVERED
October 1, 1190 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Selective Breeding for Ethanol Tolerance

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	K.A. Grant	Senior Staff Fellow	USP, NIAAA
Others:	P.L. Hoffman	Section Chief	LPPS, NIAAA

COOPERATING UNITS *(if any)*

None

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☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This experiment is a continuing project examining if tolerance to ethanol following chronic exposure has a large genetically determined component. Over the past year 3 selected generations of both replicate lines and rats from the two control lines (1200 rats in total) have been tested and bred for the degree of tolerance to ethanol. Presently, the fifth generation of the High Tolerant (AHT, BHT) and Low Tolerance (ALT, BLT) lines have been tested. Based on the results of previous studies using an ethanol related trait as the phenotype, selected lines do not clearly start to separate until after the fifth generation. Thus, this project is still in the early stages, and an evaluation of the success of the project is premature. However, the B replicate lines have remained separate for the last 4 generations, while the A replicate line remain close in their tolerant scores. There also appears to be less variance in the degree of tolerance acquired across the generations tested.

This project has been terminated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00707-03 LPPS

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Behavioral Pharmacology of Ethanol

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	K.A. Grant	Senior Staff Fellow	LPPS, NIAAA
Others:	G. Colombo	Visiting Fellow	LPPS, NIAAA

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Laboratory of Physiologic and Pharmacologic Studies

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☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

In our continuing effort to characterize the discriminative stimulus effects of ethanol, we studied the ability of various receptor agonists and antagonists to substitute for or antagonize the ethanol cue. Blockade of the discriminative stimulus effects of ethanol in pigeons by antagonists of the 5-HT3 receptor/ion channel complex was replicated in another species, rats. The discriminative stimulus effects of competitive and uncompetitive NMDA receptor complex antagonists were found to be similar to the discriminative stimulus effects of ethanol. Agonists at the GABAA receptor complex were also found to have similar discriminative stimulus effects as ethanol. The results imply that (1) 5-HT3 mediated neurotransmission is necessary for the mediation of the discriminative stimulus effects of ethanol and that (2) antagonism of the NMDA receptor or potentiation of the GABAA receptor complex result in discriminative stimulus effects similar to those of ethanol. In a separate set of studies we have investigated the influence of vasopressin in the process of information retention using a T-maze alternation task. Rats that are homozygous or heterozygous for a gene with a single base-pair substitution in the region encoding for the hormone vasopressin (the diabetes insipidus (di) gene) were compared to rats with a normal gene for vasopressin. There is a clear difference in the amount of delay the 3 groups of rats can tolerate before their ability to alternate their responses in the T-maze is impaired. The results show that vasopressin is not necessary for performance or ability to learn the task, but is crucial for the animals to retain information. Since tolerance can be viewed as an adaptation to ethanol that is retained over time, these data support the hypothesis that vasopressin is necessary for the maintenance, but not the production, of tolerance to ethanol.

This project has been terminated.

Project Description:

Investigators:

K. Grant	Senior Staff Fellow	LPPS, NIAAA
G. Colombo	Visiting Fellow	LPPS, NIAAA

Objectives:

The goal of this project is, in general, to characterize the behavioral pharmacology of ethanol. Particular objectives are to study the discriminative and reinforcing effects of ethanol, and the process of developing and maintaining functional tolerance to ethanol. Drug discrimination procedures are used to study an animal analogue of the subjective effects of psychoactive drugs such as ethanol, as well as to study the neurotransmitter systems involved in mediating the behavioral effects of ethanol. Self-administration procedures are used to characterize the reinforcing potential of ethanol. Since some receptor-gated ion channels appear to be particularly sensitive to the actions of ethanol, they have been the first systems investigated. These include the GABA-benzodiazepine receptor complex, the NMDA subtype of quatamate receptor and the 5-HT3 subtype of serotonin receptors. To study the relative roles of these different receptor systems, the discriminative stimulus properties of pentobarbital, phencyclidine and ethanol were investigated. An additional set of studies investigated the role of vasopressin in simple memory tasks and correlated these effects with the ability of vasopressin to retain functional tolerance to correlated these effects with the ability of vasopressin to retain functional tolerance to alcohol. These studies used rats bred to contain the diabetes insipidus gene originally found in the Brattleboro rat. This gene results in the homozygous carrier lacking the functional hormone vasopressin.

Methods Employed:

Drug Discrimination

Rats maintained at 85% of free-feeding body weight are trained to respond on one of two levers in an operant chamber for the delivery of food reinforcement. The animals are then given either ethanol or water (i.e.) 20 minutes prior to the onset of a session. The animal must learn to respond differentially on either one lever or the other, based on the drug condition. Thus, the only cue dictating the correct response is the presence or absence of the internal effects of ethanol. Incorrect responses during training conditions result in resetting the ratio requirement for the correct key. Following training, various doses of ethanol or other drugs are tested under conditions in which responding on either the ethanol-associated lever or the water-associated lever is reinforced. The degree of ethanol generalization is measured by the percentage of total responses on the ethanol-appropriate lever. Nonspecific drug effects are measured by decrements in rates of responding.

Maze Studies

The animals are food restricted, and maintained at 85% of pre-experimental weight. A small portion of food is placed in one arm of a T-maze and the first trial is a forced choice. The animal is then trained to alternate between the left and right arms in order to receive food. Each trial consists of placing the rat in the runway, then allowing it to choose and enter a side. Once the rat completes 9 of 11 trails correctly, a delays of 30 sec increments are imposed between completion of trials.

Major Findings:

The 5-HT3 antagonists MDL 72222 (3-10 mg/kg) completely blocked the discriminative stimulus effects of 1.0, 1.5 or 2.0 g/kg ethanol in rats. Other control drugs tested were the antipsychotic clozapine (0.1-3.0mg/kg) and the 5-HT2 antagonist ketanserin (5.6-17mg/kg); neither of which blocked the discriminative stimulus effects of ethanol. MDL 72222 administration did not block the discriminative stimulus effects of pentobarbital whether the animal was trained with pentobarbital or whether pentobarbital was tested in an ethanol-training animal. These results indicate a selective effect of MDL 72222 on blocking ethanol's discriminative stimulus effects.

The NMDA antagonists MK-801, PCP and ADCI all have discriminative stimulus effects similar to ethanol. The competitive NMDA receptor antagonists CGS 19755 and CPPene only partially substitute for the ethanol cue. These results imply that ethanol may exert is antagonism of the NMDA receptor complex by affecting the channel rather than at the NMDA recognition site.

Steroid agonists of the GABAA receptor, alloprenenalone and THDOC also have discriminative stimulus effects that are similar to ethanol. Thus, the ability of ethanol to potentiate the action of GABA can be recognized by the animal as ethanol-like when treated with naturally occurring GABA agonists.

The T-maze data show that rats homozygous for the diabetes insipidus gene can perform only at delays less than 210 sec, heterozygous rats can perform at delays less than 300 sec and normal rats can perform at delays of up to 540 sec. These data demonstrate that functional vasopressin is important in the maintenance of functional tolerance to ethanol and the retention of information in the alternation task.

Significance to Biomedical Research and the Program of the Institute:

The drug discrimination procedure allows for the evaluation of receptor mechanisms involved with mediating a behavioral effect of ethanol. This information can then be tested in other behavioral paradigms where ethanol is known to have effects. For example, there is a strong correlation between the discriminative stimulus effects of abused drugs and their reinforcing effects. Thus, information from the discrimination procedure may also suggest pharmacological manipulations to alter ethanol's reinforcing effects. As well, the process of retaining or accelerating the loss of tolerance to ethanol may have profound effects on the self-administration of ethanol. The same receptors sensitive to ethanol's effects in drug discrimination procedures or the maintenance of tolerance may affect other behavioral effects of ethanol such as anxiolytic, motor, amnestic (etc) effects. Information on how these effects of ethanol are mediated and how to alter them may be useful in lessening the impact of ethanol upon behavior, hopefully decreasing both the biomedical and psychological toxicity.

Proposed Course:

Over the next year, we have planned to begin a series of self-administration studies using both oral and intravenous routes of administration in order to investigate the reinforcing effects of ethanol. The reinforcing effects of ethanol are hypothesized to maintain excessive consumption. Since there is a good correlation between the reinforcing and the discriminative stimulus effect of a drug, information gathered from the drug discrimination studies can be directly applied to the study of the reinforcing effects. This procedure will be used to help evaluate possible inhibitors of ethanol consumption.

Publications:

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Tabakoff B, Rabe CS, Grant KA, Hudspith M, Hoffman PL. Ethanol and the NMDA receptor: Insights into ethanol pharmacology. In: Koob G, Lewis M, Meyer R, Paul SM, eds. Alcohol Reinforcement. Boston: Birkhauser, 1991:93-106.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00706-03 LPPS

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Effects of Ethanol on NMDA-Medicated Neuronal Function

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboretory, and institute affiliation)*

P.I.:	C.S. Rabe	Special Volunteer	USP, NIAAA
	B. Tabakoff	Scientific Director	NIAAA
Others:	J. Contrera	Special Volunteer	USP, NIAAA
	P.L. Hoffman	Section Chief	LPPS, NIAAA

COOPERATING UNITS *(if any)*

None

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☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

One potential mechanism by which ethanol might produce its characteristic central nervous system (CNS) depression is through inhibition of excitatory transmission. Glutamate is the most abundant excitatory neurotransmitter present in the CNS. Therefore, we examined the effect of ethanol on glutamate-mediated transmission in primary cultures of cerebellar neurons. Ethanol was a potent and selective inhibitor of the actions of glutamate at the NMDA receptor subtype, and was less effective at the kainate receptor in these cells. To define the site of action of ethanol at the NMDA receptor, interactions of ethanol with several modulators of receptor function were examined. Ethanol-induced inhibition of NMDA-stimulated calcium influx was additive with inhibition produced by phencyclidine (PCP) or magnesium ion, indicating that ethanol does not act at a site within the receptor-gated ion channel. Ethanol also did not alter the EC_{50} for NMDA stimulation of calcium uptake, suggesting no direct interaction with the NMDA binding site. However, glycine, which acts at a strychnine-insensitive site to enhance responses to NMDA, and has been suggested to be a co-agonist at the NMDA receptor, was found, at high concentrations, to reverse ethanol-induced inhibition of NMDA-stimulated calcium uptake. Structure-activity studies showed that other amino acids that act as agonists at the glycine site also reversed ethanol inhibition. The data suggest that ethanol may interfere with the concerted actions of glutamate and glycine at the NMDA receptor. The specificity of ethanol's action was investigated by comparing effects of barbiturates and benzodiazepines. Barbiturates were more effective at inhibiting kainate- than NMDA-stimulated calcium influx, while flurazepam had no effect. The differential profile of drug effects at excitatory amino acid receptors may contribute to specific pharmacological actions of these drugs.

This project has been terminated.

Publications:

Tabakoff B, Rabe CS, Grant KA, Hudspith M, Hoffman PL. Ethanol and the NMDA receptor: Insights into ethanol pharmacology. In: Koob G, Lewis M, Meyer R, Paul SM, eds. Alcohol Reinforcement. Boston: Birkäuser, 1991.

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